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BACTERIOLOGICAL EXAMINATION OF DRINKING WATER. TRAINING COURSE MANUAL IN WATER SUPPLY AND SEA RESOURCES

National Center for Urban and Industrial Health
Cincinnati, Ohio

January 1969

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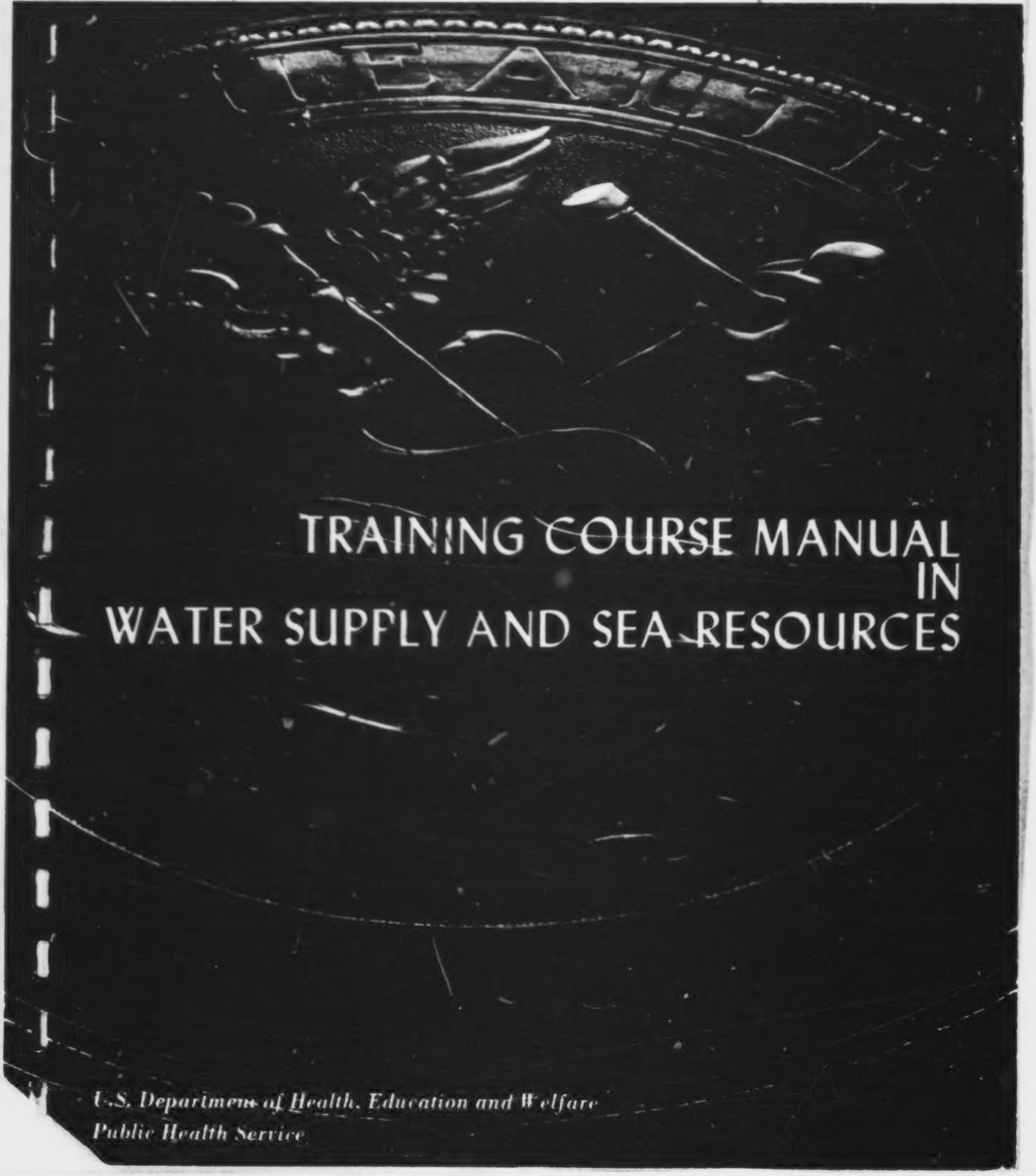
BACTERIOLOGICAL EXAMINATION OF DRINKING WATER



TRAINING COURSE MANUAL IN WATER SUPPLY AND SEA RESOURCES

U.S. Department of Health, Education and Welfare
Public Health Service

PB 216 490

BACTERIOLOGICAL EXAMINATION OF DRINKING WATER

This course is designed to provide microbiological training for individuals directly involved in the bacteriological examination of drinking water. The training course manual has been prepared especially for the trainees and should not be included in reading lists or periodicals as generally available.

Conducted by
Water Supply and Sea Resources Training Operations
TRAINING PROGRAM

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Consumer Protection and Environmental Health Services

Environmental Control Administration
National Center for Urban and Industrial Health
Cincinnati, Ohio 45202

January 1969

55

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BACTERIOLOGICAL EXAMINATION OF DRINKING WATER

January 20-24, 1969

AGENDA

Course Leader: D.A. Hunt

Moderator: J. Dennis

DAY AND TIME	SUBJECT	OUTLINE NO.	SPEAKER
Monday, January 20			
8:15 - 8:30	Registration, 4676 Columbia Parkway Room B-56		J.M. Dennis
8:30 - 8:45	Welcome		J.P. Sheehy
8:45 - 9:00	Course Objectives		D.A. Hunt
9:00 - 10:00	Bacteriological Indicators of Water Pollution	1	D.A. Hunt
10:00 - 10:15	Break		
10:15 - 11:15	Membrane Filter in Water Bacteriology	5	R. Russomanno
11:15 - 12:15	Examination of Water for Coliform and Fecal Streptococcus Groups (Multiple Dilution Tube (MPN) Methods)	6	D.A. Hunt
12:15 - 1:15	Lunch		
1:15 - 1:30	Laboratory Briefing		Staff
1:30 - 4:30	Laboratory		
Tuesday, January 21			
8:30 - 9:30	Interstate Carrier Water Supply Program	2	R.D. Lee
9:30 - 9:45	Break		
9:45 - 10:45	Media Preparation and Equipment	4	A.R. Brazis
10:45 - 11:45	Membrane Filter Laboratory and Field Procedures	7	R. Russomanno
11:45 - 12:30	Sampling, Transportation and Storage	3	J.M. Dennis
12:30 - 1:30	Lunch		
1:30 - 1:45	Laboratory Briefing		Staff
1:45 - 4:30	Laboratory		
Wednesday, January 22			
8:30 - 9:30	Basic Statistics	11	J.H. Parker
9:30 - 9:45	Break		
9:45 - 10:45	MPN-Theory, Use of Tables, and Reporting	12	J.H. Parker
10:45 - 11:45	Water Suitability Test	9	O.W. Kaufmann
11:45 - 12:30	Water Quality Behavior in Reserviors	15	J.M. Symons
12:30 - 1:30	Lunch		
1:30 - 2:30	Chlorination and Other Disinfection Practices	19	J.M. Dennis

AGENDA

DAY AND TIME	SUBJECT	OUTLINE NO.	SPEAKER
Wednesday, January 22			
2:30 - 4:30	Laboratory		Staff
Thursday, January 23			
8:30 - 9:30	Responsibilities of a Survey Officer	13	D. Van Donsel
9:30 - 9:45	Break		
9:45 - 10:45	Algae and Actinomycetes in Water Supplies	16	C.M. Palmer
10:45 - 11:45	Animal Plankton	17	R.M. Sinclair
11:45 - 12:30	Selection of Sample Filtration Volumes	8	H.L. Jeter
12:30 - 1:30	Lunch		
1:30 - 2:30	Gram Stain Procedures and Use of the Microscope	10	O.W. Kaufmann
2:30 - 4:30	Laboratory		Staff
Friday, January 24			
8:30 - 9:30	Indicator-Pathogen Relationships in Tributary Streams	18	R.M. Twedt
9:30 - 9:45	Break		
9:45 - 10:45	Proposed Revisions in the 13th Edition of Standard Methods	14	E. Geldreich
10:45 - 11:30	Discussion of Data		Staff
11:30 - 12:00	Course Critique and Closing		J. Dennis

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CONTENTS

		Outline Number
SECTION I	GENERAL	
	Bacteriological Indicators of Water Pollution	1
	Interstate Carrier Water Supply Program	2
	Sampling Transportation and Storage	3
	Media Preparation and Equipment	4
	The Membrane Filter in Water Bacteriology	5
SECTION II	PROCEDURES	
	Examination of Water for Coliform and Fecal Streptococcus Groups (Multiple Dilution Tube (MPN) Methods)	6
	Membrane Filter Laboratory and Field Procedures	7
	Selection of Sample Filtration Volumes	8
	Water Suitability Test	9
	Gram Stain Procedures and Use of Microscope	10
SECTION III	STATISTICS	
	Basic Statistics - Part I and Part II	11
	MPN Theory Use of Tables Reporting	12
SECTION IV	LABORATORY EVALUATION	
	Responsibilities of a Survey Officer	13
	Proposed Revisions in the 13th Edition of Standard Methods	14
SECTION V	WATER QUALITY FACTORS	
	Water Quality Behavior in Reservoirs	15
	Algae and Actinomycetes in Water Supplies	16
	Animal Plankton	17
	Indicator-Pathogen Relationships in Tributary Streams	18
	Chlorination and Other Disinfection Practices	19

		Outline Number
SECTION VI	LABORATORY INSTRUCTIONS	
	Exercise I MPN Presumptive and Confirmed Test	20
	Exercise II Standard Plate Count	21
	Exercise III Completed and Fecal Coliform Tests	22
	Exercise IV Membrane Filter Procedures	23

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BACTERIOLOGICAL INDICATORS OF WATER POLLUTION

Part 1. General Concepts

I INTRODUCTION

A Bacterial Indication of Pollution

1 In the broadest sense, a bacterial indicator of pollution is any organism which, by its presence, would demonstrate that pollution has occurred, and often suggest the source of the pollution.

2 In a more restrictive sense, bacterial indicators of pollution are associated primarily with demonstration of contamination of water, originating from excreta of warm-blooded animals (including man, domestic and wild animals, and birds).

B Implications of Pollution of Intestinal Origin

1 Intestinal wastes from warm-blooded animals regularly include a wide variety of genera and species of bacteria. Among these the coliform group may be listed, and species of the genera Streptococcus, Lactobacillus, Staphylococcus, Proteus, Pseudomonas, certain spore-forming bacteria, and others.

2 In addition, many kinds of pathogenic bacteria and other microorganisms may be released in wastes on an intermittent basis, varying with the geographic area, state of community health, nature and degree of waste treatment, and other factors. These may include the following:

a Bacteria: Species of Salmonella, Shigella, Leptospira, Brucella, Mycobacterium, and Vibrio comma.

b Viruses: A wide variety, including that of infectious hepatitis, Polioviruses, Coxsackie virus, ECHO viruses (enteric cytopathogenic human orphan -- "viruses in search of a disease"), and unspecified viruses postulated to account for outbreaks of diarrheal and upper respiratory diseases of unknown etiology, apparently infective by the water-borne route.

c Protozoa: Endamoeba histolytica

3 As routinely practiced, bacterial evidence of water pollution is a test for the presence and numbers of bacteria in wastes which, by their presence, indicate that intestinal pollution has occurred. In this context, indicator groups discussed in subsequent parts of this outline are as follows:

a Coliform group and certain subgroupings.

b Fecal streptococci and certain subgroupings

c Miscellaneous indicators of pollution.

4 Evidence of water contamination by intestinal wastes of warm-blooded animals is regarded as evidence of health hazard in the water being tested.

II PROPERTIES OF AN IDEAL INDICATOR OF POLLUTION

A An "ideal" bacterial indicator of pollution should:

1 Be applicable in all types of water;

2 Always be present in water when pathogenic bacterial constituents of fecal contamination are present. Ramifications of this include --

a Its density should have some direct relationship to the degree of fecal pollution, b It should have greater survival time in water than enteric pathogens,

c It should disappear rapidly from water following the disappearance of pathogens, either through natural or man-made processes, and

d It always should be absent in a bacteriologically safe water.

3 Lend itself to routine quantitative testing procedures without interference or confusion of results due to extraneous bacteria; and,

4 Be harmless to man and other animals.

B In all probability, an "ideal" bacterial indicator does not exist. The discussion of bacterial indicators of pollution in the following parts of this outline include consideration of the merits and limitations of each group, with their applications in evaluating bacterial quality of water.

III APPLICATIONS OF TESTS FOR POLLUTION INDICATORS

A Tests for Compliance with Bacterial Water Quality Standards

1 Potability tests on drinking water to meet Interstate Quarantine or other standards of regulatory agencies.

2 Determination of bacterial quality of environmental water for which quality standards may exist, such as shellfish waters, recreational waters, water resources for municipal or other supplies.

3 Tests for compliance with established standards in cases involving the protection or prosecution of municipalities, industries, etc.

B Treatment Plant Process Control

1 Water treatment plants

2 Wastewater treatment plants

C Water Quality Surveys

1 Determination of intestinal pollution in surface water to determine type and extent of treatment required for compliance with standards.

2 Tracing sources of pollution.

3 Determination of effects on bacterial flora, due to addition of organic or other wastes.

D Special studies, such as

1 Tracing sources of intestinal pathogens in epidemiological investigations.

2 Investigations of problems due to the Sphaerotilus group.

3 Investigations of bacterial interference to certain industrial processes, with respect to such organisms as Pseudomonas, Achromobacter, or others.

IV SANITARY SURVEY

The laboratory bacteriologist is not alone in evaluation of indication of water pollution of intestinal origin. On-site study (Sanitary Survey) of the aquatic environment and adjacent areas, by a qualified person, is a necessary collateral study with the laboratory work and frequently will reveal information regarding potential bacteriological hazard which may or may not be demonstrated through laboratory findings from a single sample or short series of samples.

Part 2. The Coliform Group and Its Constituents

I ORIGINS AND DEFINITION

A Background

1 In 1885, Escherich, a pioneer bacteriologist, recovered a bacterium from human feces, which he found in such numbers and consistency as to lead him to term the organism "the characteristic organism of human feces."

He named the organism *Bacterium coli-commune*. In 1895, another bacteriologist, Migula, named the organism *Escherichia coli*, which today is the official name for the type species.

2 Later work has substantiated much of the original concept of Escherich, but has shown what first was regarded as a single bacterial species is in fact a heterogeneous complex of bacterial species and species variants.

a This heterogeneous group occurs not only in human feces but representatives also are to be found in many environmental media, including sewage, surface freshwaters of all categories, in and on soils, vegetation, etc.

b The group may be subdivided into various categories on the basis of numerous biochemical and other differential tests that may be applied.

B Composition of the Coliform Group

1 Current definition

As defined in "Standard Methods for the Examination of Water and Wastewater" (12th ed): "The coliform group includes all of the aerobic and facultative anaerobic, Gram-negative, nonspore-forming rod-shaped bacteria which ferment lactose with gas formation within 48 hours at 35°C."

2 The term "coliforms" or "coliform group" is an inclusive one, including the following bacteria which may meet the definition above:

a *Escherichia coli*, *E. aurescens*, *E. freundii*, *E. intermedia*

b *Aerobacter aerogenes*, *A. cloacae*

c Biochemical intermediates between the genera *Escherichia* and *Aerobacter*

3 There is no provision in the definition of coliform bacteria for "atypical" or "aberrant" coliform strains.

a An individual strain of any of the above species may fail to meet one of the criteria of the coliform group.

b Such an organism, by definition, is not a member of the coliform group, even though a taxonomic bacteriologist may be perfectly correct in classifying the strain in one of the above species.

II SUBDIVISION OF COLIFORMS INTO "FECAL" AND "NONFECAL" CATEGORIES

A Need

Single-test differentiations between coliforms of "fecal" origin and those of "nonfecal" origin are based on the assumption that typical *E. coli* and closely related strains are of fecal origin while *A. aerogenes* and its close relatives are not of direct fecal origin. (The latter assumption is not fully borne out by investigations at this Center. See Table 1, IMViC Type --++). A number of single differential tests have been proposed to differentiate between "fecal" and "nonfecal" coliforms. Without discussion of their relative merits, several may be cited here:

B Types of Single-Test Differentiation

1 Determination of gas ratio

Fermentation of glucose by E. coli results in gas production, with hydrogen and carbon dioxide being produced in equal amounts. Fermentation of glucose by A. aerogenes results in generation of twice as much carbon dioxide as hydrogen.

Further studies suggested absolute correlation between H_2/CO_2 ratios and the terminal pH resulting from glucose fermentation. This led to the substitution of the methyl red test.

2 Methyl red test

Glucose fermentation by E. coli typically results in a culture medium having terminal pH in the range 4.2 - 4.6 (red color a positive test with the addition of methyl red indicator). A. aerogenes typically results in a culture medium having pH 5.6 or greater (yellow color, a negative test).

3 Indole

When tryptophane, an amino acid, is incorporated in a nutrient broth, typical E. coli strains are capable of producing indole (positive test) among the end-products, whereas A. aerogenes does not (negative test).

In reviewing technical literature, the worker should be alert to the method used to detect indole formation, as the results may be greatly influenced by the analytical procedure.

4 Voges-Proskauer test (acetylmethyl carbinol test)

The test is for detection of acetylmethyl carbinol, a derivative of 2, 3, butyleneglycol, as a result of glucose fermentation in the presence of peptone. A. aerogenes produces this end product (positive test) whereas E. coli gives a negative test.

a Experience with coliform cultures giving a positive test has shown a loss of this ability with storage on laboratory media for 6 months to 2-1/2 years, in 20 - 25% of cultures (105 out of 458 cultures).

b Some workers consider that all coliform bacteria produce acetylmethyl carbinol in glucose metabolism. These workers regard acetylmethyl carbinol-negative cultures as those which have enzyme systems capable of further degradation of acetylmethyl carbinol to other end products which do not give a positive test with the analytical procedure. Cultures giving a positive test for acetylmethyl carbinol lack this enzyme system.

c This reasoning leads to a hypothesis (not experimentally proven) that the change of reaction noted in certain cultures in 4.a above is due to the activation of a latent enzyme system.

5 Citrate utilization

Cultures of E. coli are unable to use the carbon of citrates (negative test) in their metabolism, whereas cultures of A. aerogenes are capable of using the carbon of citrates in their metabolism (positive test).

Some workers (using Simmons Citrate Agar) incorporate a pH indicator (brom thymol blue) in the culture medium in order to demonstrate the typical alkaline reaction (pH 8.4 - 9.0) resulting with citrate utilization.

6 Elevated temperature (Eijkman) test

a The test is based on evidence that E. coli and other coliforms of fecal origin are capable of growing and fermenting carbohydrates (glucose or lactose) at temperatures significantly higher than the body temperature of warm-blooded animals. Organisms not associated with direct fecal origin would give a negative test result, through their inability to grow at the elevated temperature.

b While many media and techniques have been proposed, EC Broth, a medium developed by Perry and Hajna, used as a confirmatory medium for 24 hours at 44.5 ± 0.5°C are the current recommended medium and method of choice. While the "EC" terminology of the medium suggests "E. coli" the worker should not regard this as a specific procedure for isolation of E. coli.

c A similar medium, Boric Acid Lactose Broth, has been developed by Levine and his associates. This medium gives results virtually identical with those obtained from EC Broth, but requires 48 hours of incubation.

d Elevated temperature tests require incubation in a water bath. The temperature conforms to that used in International Standards (45.5 ± 0.5°C). Various workers have urged use of temperatures ranging between 43.0°C and 46.0°C. Most of these recommendations have provided a tolerance of ± 0.5°C from the recommended levels. However, some workers, notably in the Shellfish Program of the Public Health Service, stipulate a temperature of 45.5°C with a tolerance of ± 0.1°C. This requires use of a water bath with forced circulation.

e The reliability of elevated temperature tests is influenced by the time required for the newly-inoculated cultures to reach the designated incubation temperature. Critical workers insist on placement of the cultures in the water bath within 30 minutes, at most, after inoculation.

7 Other tests

Numerous other tests for differentiation between coliforms of fecal vs. nonfecal origin have been proposed. Current studies suggest little promise for the following tests in this application: uric acid test, cellobiose fermentation, gelatin liquefaction, production of hydrogen sulfide, sucrose fermentation, and others.

C IMViC Classification

1 In 1938, Parr reported on a review of a literature survey on biochemical tests used to differentiate between coliforms of fecal vs. nonfecal origin. A summary follows:

Test	No. of times used for differentiation
Voges-Proskauer reaction	22
Methyl red test	20
Citrate utilization	20
Indole test	15
Uric acid test	6
Cellobiose fermentation	4
Gelatin liquefaction	3
Eijkman test	2
Hydrogen sulfide production	1
Sucrose fermentation	1
a-Methyl-d-glucoside fermentation	1

2 Based on this summary and on his own studies, Parr recommended utilization of a combination of tests, the indole, methyl red, Voges-Proskauer, and the citrate utilization tests for this differentiation. This series of reactions is designated by the mnemonic "IMViC". Using this scheme, any coliform culture can be described by an "IMViC Code" according to the reactions for each culture. Thus, a typical culture of

E. coli would have a code ++--, and a typical A. aerogenes culture would have a code --++.

3 Groupings of coliforms into fecal, nonfecal, and intermediate groups, as shown in "Standard Methods for the Examination of Water and Wastewater" are shown at the bottom of this page.

D Need for Study of Multiple Cultures

All the systems used for differentiation between coliforms of fecal vs. those of nonfecal origin require isolation and study of numerous pure cultures. Many workers prefer to study at least 100 cultures from any environmental source before attempting to categorize the probable source of the coliforms.

III NATURAL DISTRIBUTION OF COLIFORM BACTERIA

A Sources of Background Information

Details of the voluminous background of technical information on coliform bacteria recovered from one or more environmental media are beyond the scope of this discussion. References 2, 3, and 4 of this outline are suggested routes of entry for workers seeking to explore this topic.

B Recent Studies on Coliform Distribution

1 Since 1960, workers at this Center have engaged in a continuing study of the natural distribution of coliform bacteria and an evaluation of procedures for differentiation between coliforms of fecal vs. probable nonfecal origin. Results of this work have special significance because:

a Rigid uniformity of laboratory methods have been applied throughout the series of studies,

b Studies are based on massive numbers of cultures, far beyond any similar studies heretofore reported,

c A wider variety of environmental and biological sources is being studied than in any previous series of reports,

d All studies are based on freshly recovered pure culture isolates from the designated sources, and

e All studies are based on cultures recovered from the widest feasible geographic range, collected at all seasons of the year. It is believed that no more representative series of studies has been made or is in progress.

Organism	Indole	Methyl red	Voges-Proskauer	Citrate
E. coli, Variety I	+	+	-	-
Variety II	-	+	-	-
E. freundii (Intermediates)				
Variety I	-	+	-	±
Variety II	+	+	-	+
A. aerogenes				
Variety I	-	-	+	±
Variety II	±	-	+	+

2 Distribution of Coliform Types

Table 1 shows the consolidated results of coliform distributions from various biological and environmental sources.

a The results of these studies show a high order of correlation between known or probable fecal origin and the typical E. coli IMViC code (++--). On the other hand, human feces tend to include appreciable numbers of A. aerogenes and other IMViC types, which some regard as "nonfecal" segments of the coliform group.

b The majority of coliforms attributable to excretal origin tend to be limited to a relatively small number of the possible IMViC codes; on the other hand, coliform bacteria recovered from undisturbed soil, vegetation, and insect life represent a wider range of IMViC codes than fecal sources, without clear dominance of any one type.

c The most prominant IMViC code from nonfecal sources is the intermediate type, -+-+, which accounts for almost half the coliform cultures recovered from soils, and a high percentage of those recovered from vegetation and from insects. It would appear that if any coliform segment could be termed a "soil type" it would be IMViC code -+-+.

d It should not be surprising that cultures of typical E. coli are recovered in relatively smaller numbers from sources judged, on the basis of sanitary survey, to be unpolluted. There is no known way to exclude the influence of limited fecal pollution from small animals and birds in such environments.

e The distribution of coliform types from human sources should be regarded as a representative value for large numbers of sources. Investigations have shown that there can be large differences in the distribution of IMViC types from person to person, or even from an individual.

3 Differentiation between coliforms of fecal vs. nonfecal origin

Table 2 is a summary of findings of SEC workers, based on a number of different criteria for differentiating between coliforms of fecal origin and those from other sources.

a IMViC type ++-- is a measurement of E. coli, Variety I, and appears to give reasonably good correlation between known or highly probable fecal origin and doubtful fecal origin.

b The combination of IMViC types, ++--, +---, and -+--, gives improved identification of probable fecal origin, and appears also to exclude most of the coliforms not found in excreta of warm-blooded animals in large numbers.

c While the indole, methyl red, Voges Proskauer, and citrate utilization tests, each used alone, appear to give useful answers when applied only to samples of known pollution from fecal sources, the interpretation is not as clear when applied to coliforms from sources believed to be remote from direct fecal pollution.

d The elevated temperature test gives excellent correlation with samples of known or highly probable fecal origin. The presence of smaller, but demonstrable, percentages of such organisms in environmental sources not interpreted as being polluted could be attributed largely to the warm-blooded wildlife in the area, including birds, rodents, and other small mammals.

e The elevated temperature test yields results equal to those obtained from the total IMViC code. It has marked advantages in speed, ease and simplicity of performance, and yields quantitative results for each water sample. Therefore, it is regarded, as the method of choice for differentiation between coliforms of probable direct fecal origin and those

Table 1. COLIFORM DISTRIBUTION BY IMViC TYPES AND ELEVATED TEMPERATURE TEST FROM ENVIRONMENTAL AND BIOLOGICAL SOURCES

IMViC type	Vegetation		Insects		Soil				Fecal sources				Poultry	
					Undisturbed		Polluted		Human		Livestock			
	No. strains	% of total	No. strains	% of total	No. strains	% of total	No. strains	% of total	No. strains	% of total	No. strains	% of total	No. strains	% of total
++--	128	10.6	134	12.4	131	5.6	536	80.6	3932	87.2	2237	95.6	1857	97.9
--++	237	19.7	113	10.4	443	18.8	13	2.0	245	5.4	0	<0.1	1	0.1
-+--	23	1.9	0	<0.1	78	3.3	1	0.2	99	2.2	14	0.6	20	1.1
+++-	2	0.2	0	<0.1	7	0.3	0	<0.1	106	2.4	59	2.5	0	<0.1
-+-+	168	14.0	332	30.6	1131	48.1	87	13.0	50	1.1	1	<0.1	5	0.3
++-+	116	9.6	118	10.9	87	3.7	22	3.3	35	0.8	27	1.2	11	0.6
-+++	32	2.7	28	2.6	181	7.7	5	0.7	21	0.5	0	<0.1	0	<0.1
++++	291	24.2	254	23.4	159	6.8	0	<0.1	6	0.1	0	<0.1	0	<0.1
+-++	88	7.3	46	4.2	67	2.9	0	<0.1	14	0.2	0	<0.1	0	<0.1
---+	87	7.2	42	3.9	4	0.2	1	0.2	2	<0.1	0	<0.1	0	<0.1
-++-	5	0.4	0	<0.1	1	<0.1	0	<0.1	0	<0.1	0	<0.1	0	<0.1
--+-	19	1.6	0	<0.1	53	2.3	0	<0.1	0	<0.1	0	<0.1	0	<0.1
+-+-	2	0.2	0	<0.1	6	0.3	0	<0.1	0	<0.1	0	<0.1	0	<0.1
+--+	5	0.4	8	0.7	0	<0.1	0	<0.1	0	<0.1	0	<0.1	0	<0.1
+---	0	<0.1	9	0.8	0	<0.1	0	<0.1	2	<0.1	0	<0.1	2	<0.1
Total	1203		1084		2348		665		4512		2339		1896	
No. EC +	169*		162*		216		551		4349		2309		1765	
% EC +	14.1*		14.9*		9.2		82.9		96.4		98.7		93.0	
	*120 of these were ++--, 15 --++, 11 -+--		*129 of these were ++--, 27 -+-+, 5 ++-+											

Table 2. COMPARISON OF COLIFORM STRAINS ISOLATED FROM WARM-BLOODED ANIMAL FECES, FROM UNPOLLUTED SOILS AND POLLUTED SOILS WITH USE OF THE IMViC REACTIONS AND THE ELEVATED TEMPERATURE TEST IN EC MEDIUM AT 44.5°C (± 0.5°)

Test	Warm-blooded animal feces	Soil: Unpolluted	Soil: Polluted	Vegetation	Insects
+ + - -	91.8%	5.6%	80.6%	10.6%	12.4%
+ + - -, + - - - and - + - -	93.3%	8.9%	80.7%	12.5%	13.2%
Indole positive	94.0%	19.4%	82.7%	52.5%	52.4%
Methyl red positive	96.9%	75.6%	97.9%	63.6%	79.9%
Voges-Proskauer positive	5.1%	40.7%	97.3%	56.3%	40.6%
Citrate utilizers	3.6%	88.2%	19.2%	85.1%	86.7%
Elevated temperature (EC) positive	96.4%	9.2%	82.9%	14.1%	14.9%
Number of cultures studied	8,747	2,348	665	1,203	1,084

Total Pure Cultures Studied: 14,047

which may have become established in the bacterial flora of the aquatic or terrestrial habitat.

IV EVALUATION OF COLIFORMS AS POLLUTION INDICATORS

A The Coliform Group as a Whole

1 Merits

a The absence of coliform bacteria is evidence of a bacteriologically safe water.

b The density of coliforms is roughly proportional to the amount of excretal pollution present.

c If pathogenic bacteria of intestinal origin are present, coliform bacteria also are present, in much greater numbers.

d Coliforms are always present in the intestines of humans and other warm-blooded animals, and are eliminated in large numbers in fecal wastes.

e Coliforms are more persistent in the aquatic environment than are pathogenic bacteria of intestinal origin.

f Coliforms are generally harmless to humans and can be determined quantitatively by routine laboratory procedures.

2 Limitations

a Some of the constituents of the coliform group have a wide environmental distribution in addition to their occurrence in the intestines of warm-blooded animals.

b Some strains of the coliform group may grow in polluted waters ("aftergrowth"), thereby adding to the difficulty of evaluating a pollution situation in the aquatic environment. Members of the A. aerogenes section of the coliform are commonly involved in this kind of problem.

c Because of occasional aftergrowth problems, the age of the pollution may be difficult to evaluate under some circumstances.

d Tests for coliforms are subject to interferences due to other kinds of bacteria. False negative results sometimes occur when species of Pseudomonas are present. False positive results sometimes occur when two or more kinds of non-coliforms produce gas from lactose, when neither can do so alone (synergism).

B The Fecal Coliform Component of the Coliform Group (as determined by elevated temperature test)

1 Merits

a The majority (over 95%) of the coliform bacteria from intestines of warm-blooded animals grow at the elevated temperature.

b These organisms are of relatively infrequent occurrence except in association with fecal pollution.

c Survival of the fecal coliform group is shorter in environmental waters than for the coliform group as a whole. It follows, then, that high densities of fecal coliforms is indicative of relatively recent pollution.

d Fecal coliforms generally do not multiply outside the intestines of warm-blooded animals. In certain high-carbohydrate wastes, such as from the sugar beet refineries, exceptions have been noted.

2 Limitations

a Feces from warm-blooded animals include some (though proportionately low) numbers of coliforms which do not yield a positive fecal coliform test when the elevated temperature test is used as the criterion of differentiation.

b There is at present no established correlation between ratios of total coliforms/fecal coliforms in establishing sanitary quality of water.

In domestic sewage, the fecal coliform density commonly is 10-30% that of the total coliform density, and sometimes may be greater. In environmental waters relatively free from recent pollution, the fecal coliform density may range commonly from 1-10% that of the total coliforms. There are, however, too many variables relating to water borne wastes and surface water run-off to permit sweeping generalization on the numerical relationships between fecal- and total coliforms.

c At this time, little is known about the survival of fecal coliforms in polluted waters compared with that of enteric pathogenic bacteria. In recent pollution studies, species of Salmonella have been found in the presence of 220 fecal coliforms per 100 ml (Spino), and 110 fecal coliforms per 100 ml (Brezenski, Raritan Bay Project).

V APPLICATIONS OF COLIFORM TESTS

A Current Status in Official Tests

1 The coliform group is designated, in "Standard Methods for the Examination of Water and Wastewater" (12th ed., 1965), through the Completed Test MPN procedure as the official test for

bacteriological potability of water. The Confirmed Test MPN procedure is accepted where it has been demonstrated, through comparative tests, to yield results equivalent to the Completed Test. The membrane filter method also is accepted for examination of waters subject to interstate regulation.

2 The 12th edition of Standard Methods has introduced a standard test for fecal coliform bacteria. It is emphasized that this is to be used in pollution studies, and does not apply to the evaluation of water for potability.

B Applications

1 Tests for the coliform group as a whole are used in official tests to comply with interstate drinking water standards, state standards for shellfish waters, and in most, if not all, cases where bacterial standards of water quality have been established for such use as in recreational or bathing waters, water supplies, or industrial supplies.

2 The fecal coliform test has application in water quality surveys, as an adjunct to determination of total coliform density. The fecal coliform test is being used increasingly in all water quality surveys.

3 It is emphasized that no responsible worker advocates substitution of a fecal coliform test for total coliforms in evaluating drinking water quality.

Part 3. The Fecal Streptococci

I DEFINITIONS

A A fecal streptococcus as used in this discussion refers to any streptococcus commonly found in significant numbers in the feces of human or other warm-blooded animals. Other terms used are "enterococci" or "Group D streptococci."

B Enterococci are characterized by specific biochemistry. Serological procedures differentiate the Group D streptococci. Although they overlap, the three groups, fecal streptococcus enterococcus, and Group D streptococcus, are not synonymous. Because our emphasis is on indicators of unsanitary origin, fecal streptococcus is the more appropriate term.

C A rigid definition of the fecal streptococcus group is not possible with our present knowledge. The British Ministry of Health (1956) defines the organisms as "Gram-positive" cocci, generally occurring in pairs or short chains, growing in the presence of bile salt, usually capable of development at 45°C, producing acid but not gas in mannitol and lactose, failing to attack raffinose, failing to reduce nitrate to nitrite, producing acid in litmus milk and precipitating the casein in the form of a loose, but solid curd, and exhibiting a greater resistance to heat, to alkaline conditions and to high concentrations of salt than most vegetative bacteria". However, it is pointed out that "streptococci departing in one or more particulars from the type species cannot be disregarded in water."

D Some workers consider that growth at 45°C and multiplication in 40% bile are the most significant indications of fecal origin of streptococci.

E The enterococci subgroup of the fecal streptococci is one of four major groups of streptococci. The first group, not growing at 10°C or 45°C, pathogenic for man and animals, causes total lysing of red blood cells, giving them the common name of beta hemolytic streptococci.

F A second group, or Viridans group, not growing at 10°C, growth at 45°C, is an intermediate group, some members resembling group 1 and others, the third group, growing at 10°C and 45°C, the enterococci. Beside their fecal origin, the enterococci typically exhibit virorous biochemical reactions. The fourth, growing at 10°C and not at 45°C, or lactic acid group, is a common harmless inhabitant of plants and dairy products.

Examples of specific strains or species representative of each group are:

1 Streptococcus pyogenes, cause of scarlet fever;

2 Streptococcus bovis, from cow manure;

3 Streptococcus fecalis, from human feces; and

4 Streptococcus lactis, from cheese.

G For special purposes, strains of individual fecal streptocci are identified by biochemical tests. The techniques are discussed elsewhere. Strains or species likely to be fecal streptococci are: Streptococcus fecalis, S. fecalis variety liquefaciens, S. fecalis variety zymogenes, S. durans, S. bovis, S. equinus, S. salivarius, and S. fecalis biotypes growing at 10°C and 45°C, usually.

II HISTORY

From their discovery to the present time the fecal streptococci appear characteristic of fecal pollution, being consistently present in both the feces of all warm-blooded animals and in the environment associated with animal discharges. As early as 1910 fecal streptococci were proposed as indicators to the Metropolitan Water Board of London. However, little progress resulted in the United States until improved methods of detection and enumeration appeared after World War II.

Renewed interest in the group as indicators began with the introduction of azide dextrose broth in 1950, (Mallmann & Seligmann, 1950). The method which is in the current edition of Standard Methods appeared soon after. (Litsky et al. 1955).

With the advent of improved methods for detection and enumeration of fecal streptococci, a significant body of technical literature has appeared.

III SIGNIFICANCE OF FECAL STREPTOCOCCAL INDICATORS

A The presence of fecal streptococci indicates the presence of warm-blooded animal pollution. Many types of wild life may harbor fecal streptococci. One study showed 71% of various mammalian species yielding enterococci. Similarly, reptiles showed 85% incidence. However, the common rodents yielded few and sporadic isolations. Wild birds also showed few individuals with enterococci. (Mundt 1963). Insects may be only accidental mechanical carriers of fecal streptococci. However, Geldreich et al. (1964), found high numbers in some insects.

B Evidence indicates that fecal streptococci do not occur in pure water or virgin soil.

In one study, Medrek and Litsky (1960) looked specifically for occurrence of enterococci in undisturbed or virgin soil. Modern methods showed 8 out of 369 samples with enterococci. With only one exception, the presence of enterococci in a given soil sample was associated with the presence of coliforms. The presence of indicators in a given soil sample is considered indicative of chance pollution of the sampling area by animal or bird droppings or drainage.

C Fecal streptococci do not multiply in water. A recent report is illustrative (Morris and Weaver, 1954). Duplicate samples were taken of 52 polluted Kentucky wells and examined before and after storage for 24 hours. While the coliform numbers increased, decreased or changed little, the streptococcal numbers never increased. Other experiments on a variety of Louisiana surface water gave same results for streptococcal numbers. Fecal streptococci enter a water, survive for a period of time and die without multiplying.

D On the basis of laboratory data, the fecal streptococci could be expected to be more resistant to electrolytes like salt. If true, the group would have special value for salt water investigation. Further research is needed to determine this point.

E The fecal streptococci are not considered pathogenic. However, such strains as S. fecalis have been known to occur with endocarditis and other human infections. S. fecalis var. zymogenes, a beta hemolytic streptococcus normally inhabiting the human intestine, is also nonpathogenic.

The fecal streptococci are alleged causes of food-borne infections. However, the circumstantial evidence has not been supported by intensive studies.

IV LIMITATIONS

A Knowledge of relative survival times of fecal streptococci and pathogens is needed. Comparable results with many types of pathogens under many environmental conditions are needed. Insufficient time has elapsed since adequate media have been developed for evidence to accumulate. The coliforms have over 60 years of accumulated validation for their use. The fecal streptococci have had only about 10 years of serious consideration. A few comparisons exist, however.

When sewage is added to the soil, the coliforms persist for long periods. Fecal streptococci disappear rapidly. Typhoid bacilli, added experimentally with sewage, persist less time than the fecal streptococci. However, mineral-organic content of the receiving soil has much influence on persistence so that only relative rates may be determined. (Mallman and Litsky, 1950).

B Although the enterococci are considered as having their origin, in the intestines of warm-blooded animals, other sources may exist. The resistance and tolerance of these streptococci, together with their low minimum and high maximum limits of growth may not only enable them to survive but also to grow under diverse conditions of nature. Reports have appeared that growth occurs in food products, fresh plants and silage. For example, some investigations (Mundt, 1962) show that fecal streptococci of the S. fecalis and the S. liquefaciens types occur commonly on plants. However, Geldreich et al, 1963 concluded after an exhaustive study of vegetation that indicator contribution was negligible.

V COLIFORM -- FECAL COLIFORM - FECAL STREPTOCOCCUS RELATIONSHIPS

Because coliforms may derive from non-fecal sources, the fecal coliforms are a more reliable standard of comparison with streptococcal densities. Even so, variation in known ratios is marked.

A Variation may arise from different methods, different species, different individuals, and different geographic locations.

B Species differences are the main cause of different fecal streptococci-fecal coliform ratios. Table 3 compares fecal streptococcus and fecal coliform counts for different species. Even though individuals vary widely, masses of individuals in a species have characteristic proportion of indicators. In intestinal wastes of human origin, the ratio of number of fecal coliforms to number of fecal streptococci tends to be considerably greater than two-to-one. In comparison, when such ratios are determined for intestinal wastes from non-human animal sources, the values tend to be markedly less than one-to-one.

C Determination of specific strains of fecal streptococci sometimes is useful. Besides ratio of indicators, the specific types of fecal streptococci are more typical of some animals. Humans carry 80% or over of S. fecalis group, and characteristically the S. salivarius-mitis group. Cows and horses carry a majority of S. bovis and S. equinus groups. Other farm animals have mixtures of appreciable numbers of several groups. Fowl, however, resemble humans in their streptococcal flora. The S. salivarius group, besides being uniquely human, dies rapidly in water, giving an index of recentness of pollution

Table 3. Estimated Per Capita Contribution of Indicator Microorganisms From Some Animals*

		Average indicator density per gram of feces		Average contribution per capita per 24 hr		
Animals	Avg wt of feces/ 24 hr, wet wt, g	Fecal coliform, million	Fecal strepto-cocci, million	Fecal coliform, million	Fecal strepto-cocci, million	Ratio FC/FS
Man	150	13.0	3.0	2,000	450	4.4
Duck	336	33.0	54.0	11,000	18,000	0.6
Sheep	1,130	16.0	38.0	18,000	43,000	0.4
Chicken	182	1.3	3.4	240	620	0.4
Cow	23,600	0.23	1.3	5,400	31,000	0.2
Turkey	448	0.29	2.8	130	1,300	0.1
Pig	2,700	3.3	84.0	8,900	230,000	0.04

*Publication WP-20-3, P. 102

VI INTERPRETATION

The presence of fecal streptococci in untreated water indicates the presence of fecal pollution by warm-blooded animals.

In samples where the source and significance of the coliform group has been questioned, the presence of the streptococcus group should be interpreted as indicating at least a portion of the coliform group were derived from fecal sources.

Because of the uncertainties in die-off rates, the absence of fecal streptococci does not necessarily mean that the water is bacteriologically safe.

Part 4. Other Bacterial Indicators of Pollution

I TOTAL BACTERIAL COUNTS

A Historical

1 The early studies of Robert Koch led him to develop tentative standards of water quality based on a limitation of not more than 100 bacterial colonies per ml on a gelatin plating medium incubated 3 days at 20°C.

2 Later developments led to inoculation of samples on duplicate plating media, with one set incubated at 37°C and the other at 20°C.

a Results were used to develop a ratio between the 37°C counts and the 20°C counts.

b Waters having a predominant count at 37°C were regarded as being of probable sanitary significance, while those giving predominant counts at 20°C were considered to be of probable soil origin, or natural inhabitants of the water being examined.

B Groups Tested

There is no such thing as "total" bacterial count in terms of a laboratory determination.

1 Direct microscopic counts do not differentiate between living and dead cells.

2 Plate counting methods enumerate only the bacteria which are capable of using the culture medium provided, under the temperature and other growth conditions used as a standard procedure. No one culture medium and set of growth conditions can provide, simultaneously, an acceptable environment for all the heterogeneous, often conflicting, requirements of the total range of bacteria which may be recovered from waters.

C Utilization of Total Counts

1 Total bacterial counts, using plating methods, are useful for:

a Detection of changes in the bacterial composition of a water source,

b Process control procedures in treatment plant operations,

c Determination of sanitary conditions in plant equipment or distributional systems.

2 Serious limitations in total bacterial counts exist because:

a No information is given regarding possible or probable fecal origin of bacterial changes,

b No information of any kind is given about the species of bacteria cultivated, and

c There is no differentiation between harmless or potentially dangerous forms.

3 Status of total counts

a There is no total bacterial count standard for any of the following:

Interstate Quarantine Drinking Water Standards

APHS regulations for water potability (as shown in "Standard Methods"

Public Health Service Drinking Water Standards of 1962.

b The most widely used current application of total bacterial counts in water bacteriology today is in water treatment plants, where some workers use standard plate counts for process control and for determination of the bacterial quality of distribution systems and equipment.

c Total bacterial counts are not used in PHS water quality studies, though extensively used until the 1940's.

B Spore-Forming Bacteria (*Clostridium perfringens*, or *C. welchii*)

1 Distribution

This is one of the most widely distributed species of bacteria. It is regularly present in the intestinal tract of warm-blooded animals.

2 Nature of organism

C. perfringens is a Gram-positive, spore-forming rod. The spores cause a distinct swelling of the cell when formed. The organism is extremely active in fermentation of carbohydrates, and produces the well-known "stormy fermentation" of milk.

3 Status

The organism, when present, indicates that pollution has occurred at some time. However, because of the extremely extended viability of the spores, it is impossible to obtain even an approximation of the recency of pollution based only on the presence of *C. perfringens*.

The presence of the organism does not necessarily indicate an unsafe water.

C Tests for Pathogenic Bacteria of Intestinal Origin

1 Groups considered include *Salmonella* sp, *Shigella* sp, *Vibrio comma*, *Mycobacterium* sp, *Pasteurella* sp, *Leptospira* sp, and others.

2 Merits of direct tests:

Demonstration of any pathogenic species would demonstrate an unsatisfactory water quality, hazardous to persons consuming or coming into contact with that water.

3 Limitations

a There is no available routine procedure for detection of the full range of pathogenic bacteria cited above.

b Quantitative methods are not available for routine application to any of the above.

c The intermittent release of these pathogens makes it impossible to regard water as safe, even in the absence of pathogens.

d After detection, the public already would have been exposed to the organism; thus, there is no built-in margin of safety, as exists with tests for the coliform group.

4 Applications

a In tracing the source of pathogenic bacteria in epidemiological investigations,

b In special research projects, and

c In water quality studies concerned with enforcement actions against pollution, increasing attention is being given to the demonstration of enteric pathogenic bacteria in the presence of the bacterial indicators of pollution.

D Miscellaneous Indicators

It is beyond this discussion to explore the total range of microbiological indicators of pollution that have been proposed and investigated to some extent. Mention can be made, however, of consideration of tests for the following:

1 Bacteriophages specific for any of a number of kinds of bacteria,

2 Serological procedures for detection of coliforms and other indicators; a certain amount of recent attention has been given to applications of fluorescent antibodies in such tests;

3 Tests for Pseudomonas aeruginosa; and

4 Tests for viruses, which may persist in waters even longer than members of the coliform group.

ACKNOWLEDGEMENT:

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INTERSTATE CARRIER WATER SUPPLY PROGRAM

R. D. Lee*

BACKGROUND:

Quarantine regulations have been enforced for many years to prevent the spread of communicable diseases. Early in this century, there was concern for the spread of disease by modern means of rapid transportation. The Interstate Quarantine Regulations are intended to control the spread of disease. Control of waterborne disease through control of the water available on conveyances began in 1914. Section 72.101 states that only potable water shall be provided for drinking and culinary purposes and that water shall have been obtained from approved watering points. Section 72.102 requires the water supply to the watering point to meet the Drinking Water Standards.

(COPY OF IQR'S TO BE DISTRIBUTED)

Operations of the water supply program applicable to the IQR's.

1 **Annual inspections by states and report to PHS regional office (see attached 682).**

2 Triennial joint surveys by state and PHS personnel.

3 **Investigations into adequacy of supply - protection of source - treatment - quality control - plumbing inspections - expansion plans.**

4 **Publication of inventory (PHS #116)**

Has been a once each 5 year operation. For internal use, expect to obtain from computer every three months. Will be as up-to-date as input from field will allow.

5 **The July 1, 1968, inventory is now in the Regional Office for identification of duplications, omissions, and errors.**

6 **During joint surveys, samples for CAM, trace elements, and radiochemistry are collected.**

7 **Evaluation of laboratories is conducted by the laboratory personnel of the Basic Data Section. The SG 1963 interpretation of the standards requires bacteriological examinations to be made in approved laboratories. PHS certifies the state labs and they, in turn, certify local laboratories.**

8 **Watering point is actual point of delivery to carrier. Tap in commissary, hydrant beside railroad track - outlet on pier. Inspected by state or local personnel in most cases - by ESP in a few states. Watering point list is not a list of supplies. If provisional, asterisks are used to indicate whether point, supply, or both have deficiencies. Failure of supply to be certified prohibits the use of all watering points served by that supply. Prohibited points are not listed. Sometimes there are local repercussions when a water supply must be prohibited.**

Changes Affecting Operations

1 **Separation of watering point and water supply responsibility - first between ESP and WSSRP - now between FDA and WSSRP.**

2 **Extension of PHS interest in water supplies through the Safe Drinking Water Act of 1968 - survey of 1,500 to 2,000 water supplies - survey of all state and territorial water supply control programs - examination and analysis of samples from all water supplies.**

3 **Increased emphasis on local laboratory certification - need for presently exempt (by state law or regulation) laboratories to be certified to comply with Drinking Water Standards.**

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2-2

REPORT OF WATER SUPPLY USED ON INTERSTATE CARRIERS

FOR USE OF PHS REGIONAL OFFICE

(Location code)

1. NAME OF WATER SUPPLY FACILITY

LOCATION *(City, County, State)*

2. WATER SUPPLY OFFICIAL CONTACTED *(Name and Title)*

3. DATE THIS SURVEY MADE — MO. DAY YR.

4. DATE OF LAST PHS - STATE JOINT SURVEY — MO. DAY YR.

5. POPULATION SERVED BY DISTRIBUTION SYSTEM *(To the nearest hundred)*

6. OWNERSHIP ☐
1 = Public
2 = Private

7. SOURCE ☐
1 = Surface
2 = Ground
3 = Combination

8. TREATMENT USED -- 1 = YES, 2 = NO

☐ Disinfection ☐ Coagulation ☐ Rapid Sand Filtration ☐ Fluoridation ☐ Taste and Odor Control

☐ Lime Softening ☐ Ion Exchange ☐ Settling ☐ Iron Removal ☐ Other *(If yes describe under 13).*

9. LOCAL WATER SUPPLY PROGRAM -- 1 = YES, 2 = NO

☐ A. Is there a continuous program, such as a cross-connection survey, to detect health hazards and sanitary defects within the water distribution system?

☐ B. If there is a program such as described in "A" above are sanitary defects and health hazards being removed systematically and at a rate which is satisfactory?

☐ C. Does the community have an adequate plumbing code, such as the National Plumbing Code, or are there rules and regulations prohibiting connections or arrangements by which liquids, steams, gasses, or chemicals of unsafe, unknown, or questionable quality may be discharged or drawn into the public water supply?

☐ D. Are there provisions to enforce such code or rules and regulations on new construction?

☐ -- on major repairs and reconstruction?

☐ E. Are the principal plant operators certified or licensed under a state program?

☐ -- is such certification or licensing compulsory?

CHEMICAL ANALYSIS (mg./1) (A blank indicates no report)

10.

MO. DAY YR. Date last chemical sample taken

How often are chemical analyses made?

Alkalinity (as Ca CO$_3$)
Arsenic (.05)**(.01)*
Barium (1.0)*
Cadmium (.01)**
Chromium Hexavalent (.05)**
Cyanide (.2)** (.01)*
Fluoride
Lead (.05)**
Hardness (as Ca CO$_3$)
Selenium (.01)**

Silver (.05)**
ABS (.5)*
Chloride (250)*
Copper (1.0)*
CCE (.2)*
Iron (.3)*
Manganese (.05)*
Nitrate (NO$_3$ (45)*
Phenols (.001)*
Sulfates (250)*

TDS (500)*
Zinc (5.0)*
pH
Magnesium

*RECOMMENDED MAXIMUM **MAXIMUM CHEMICAL LIMITS --MG/L

PHYSICAL AND RADIOCHEMICAL ANALYSIS

11.
How often are physical analyses made?

PHYSICAL

Turbidity (5 s.u.)
Color (15 s.u.)
Odor (3 s.u.)
Avg. Water Temp. (°F)
Avg. Air Temp. (°F)①

RADIOCHEMICAL

SR^{90} (10 $\mu\mu c/1$)*
RA^{226} (3 $\mu\mu c/1$)*
GROSS BETA (1000 $\mu\mu c/1$)*
GROSS ALPHA
SR^{89}
I^{131}

* Limits from 1962 PHS Drinking Water Standards

PHS-682 (REV. 10-64)

2-3

BACTERIOLOGICAL EXAMINATIONS

12. ☐☐☐☐ Minimum number of samples required per month①

LABORATORIES	DATES OF MOST RECENT LABORATORY CERTIFICATIONS

NAMES OF LABORATORY CERTIFYING AUTHORITIES

			TUBE METHOD					MEMBRANE FILTER METHOD					
			☐ 10 ML. OR ☐ 100 ML. STANDARD PORTIONS										
MONTH AND YEAR		NUMBER OF SAMPLES	NUMBER	NUMBER POSITIVE ②	% POSITIVE ③	SAMPLES HAVING 3 OR MORE 10 ML. OR 5 100 ML. PORTIONS POSITIVE ④		MONTH AND YEAR		NUMBER OF SAMPLES	MEAN COLIFORM DENSITY PER 100 ML. ⑤	SAMPLES HAVING COLIFORM DENSITIES EXCEEDING 3/50, 4/100, 7/200, 13/500 MLS. ⑥	
MO.	YR.					NUMBER	% POSITIVE	MO.	YR.			NUMBER	% POSITIVE

Footnotes:

1. See Figure 1 PHS Drinking Water Standards (1962)
2. Any member of the coliform group of bacteria.
3. Should not exceed 10% per month, if 10 ml. portions are used, or 60% if 100 ml. portions are used.
4. If 10 ml. portions are used, should not exceed 5% in 20 or more samples per month nor one sample in less than 20 per month; if 100 ml. portions are used, should not exceed 20% in 5 or more samples per month nor one sample in less than 5 per month.
5. The arithmetic means density of all standard samples per month shall not exceed 1 per 100 ml.
6. Should not exceed 5% in 20 or more samples per month, nor 1 sample in less than 20 per month.
7. Maximum daily air temperature average for at least 5 years.

13. COMMENTS: *(Use additional sheet if necessary. Furnish complete description every three years)*

REVIEWING ENGINEER *(Name and Title)*	AGENCY
CLASSIFICATION RECOMMENDED 1–Approved 2–Provisionally Approved 3–Prohibited ☐	EXPIRATION DATE *(If provisionally approved)* ☐☐ MO. ☐☐ DAY ☐☐ YR.

DATE OF REVIEW ☐☐ MO. ☐☐ DAY ☐☐ YR.	SIGNATURE OF STATE HEALTH OFFICIAL	TITLE IF OTHER THAN REVIEWER

HS-682 (BACK)
. 10-64

SAMPLING TRANSPORTATION AND STORAGE

Joseph M. Dennis*

I THEORY OF SAMPLING

A sample of water can be taken at anytime, anywhere, for chemical, physical, or bacteriological analyses. The tests, however, will only indicate the quality of that particular small quantity of water. What the investigator wants to know is whether this small sample of water is representative of the larger quantity being used by the general public.

If the sample is carelessly taken in the field the results from the laboratory will be both erratic and unreliable.

One sample from a point in the water system is of little value. A long record of the water quality is desirable. The greater the variation in the chemical, physical or bacteriological content of the water the more frequent one should sample.

Streams are subject to quite rapid change in quality. As you go further down the line in the treatment processes these peaks are dampened to a considerable extent.

Chemical and physical samples can be "composited according to flow" and be more representative of the flow over the entire sampling period. This method of sampling, however, is not practical for bacteriological investigations as the quality of the water in the sample may or may not change from the time the water is collected until it is taken to the laboratory.

Therefore, samples should be taken at appropriate intervals at points where it is necessary to determine the quality and effectiveness of treatment. Many laboratories, especially those in utilities serving large population centers have permanent sampling stations located along in the treatment facilities and distribution system.

How many samples should be taken of a system to satisfy the "representative sample" requirement? Quoting from Public Health Service Drinking Water Standards of 1962:(1)

3 BACTERIOLOGICAL CHARACTERISTICS

3.1 Sampling

3.11 Compliance with the bacteriological requirements of these Standards shall be based on examinations of samples collected at representative points throughout the distribution system. The frequency of sampling and the location of sampling points shall be established jointly by the Reporting Agency and the Certifying Authority after investigation by either agency, or both, of the source, method of treatment, and protection of the water concerned.

3.12 The minimum number of samples to be collected from the distribution system and examined each month should be in accordance with the number on the graph in Figure 1, for the population served by the system.

3.13 In determining the number of samples examined monthly, the following samples may be included, provided all results are assembled and available for inspection and the laboratory methods and technical competence of the laboratory personnel are approved by the Reporting Agency and the Certifying Authority:

a Samples examined by the Reporting Agency.

b Samples examined by local government laboratories.

c Samples examined by the water works authority.

d Samples examined by commercial laboratories.

3.14 The laboratories in which these examinations are made and the method used in making them shall be subject to inspection at any time by the designated representatives of the Certifying Authority and the Reporting

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*Chief, Water Supply & Sea Resources Training Operations, Training Program, NCUI

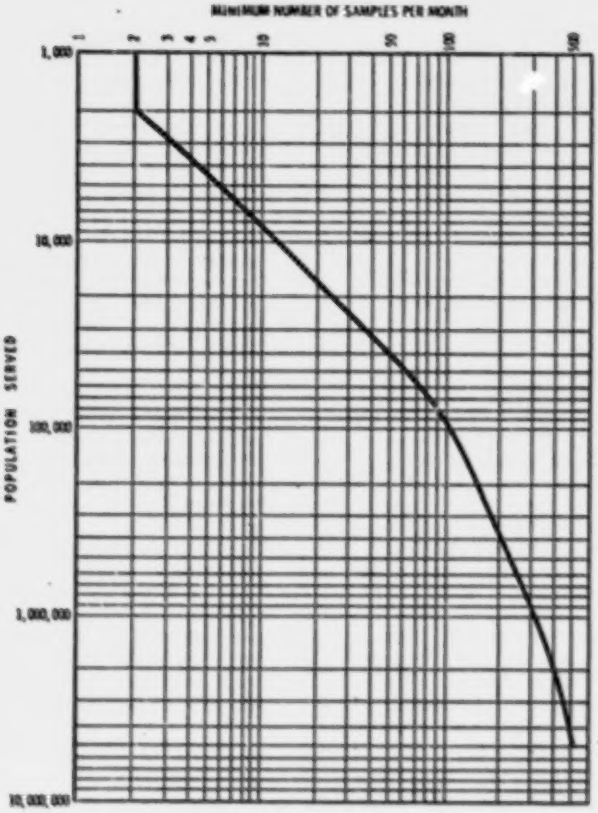

MINIMUM NUMBER OF SAMPLES PER MONTH
1
2
3
4
5
10
50
100
500
POPULATION SERVED
1,000
10,000
100,000
1,000,000
10,000,000

Figure 1

Agency. Compliance with the specified procedures and the results obtained shall be used as a basis for certification of the supply.

4 PHYSICAL CHARACTERISTICS

4.1 Sampling.

The frequency and manner of sampling shall be determined by the Reporting Agency and the Certifying Authority. Under normal circumstances samples should be collected one or more times per week from representative points in the distribution system and examined for turbidity, color, threshold odor, and taste.

5 CHEMICAL CHARACTERISTICS

5.1 Sampling.

5.11 The frequency and manner of sampling shall be determined by the Reporting Agency and the Certifying Authority. Under normal circumstances, analyses for substances listed below need be made only seminannually. If, however, there is some presumption of unfitness because of the presence of undesirable elements, compounds, or materials, periodic determinations for the suspected toxicant or material, should be made more frequently and an exhaustive sanitary survey should be made to determine the source of the pollution. Where the concentration of a substance is not expected to increase in processing and distribution, available and acceptable source water analyses performed in accordance with standard methods may be used as evidence of compliance with these Standards.

II PRACTICE

A Containers

Samples should be collected for bacteriological examination in bottles which have been cleaned and sterilized in accordance "Standard Methods for the Examination of Water and Waste Water."(2) The bottle should be large enough to hold the amount of water required for the bacteriological tests and have space at the top to allow the sample to be shaken at the laboratory before it is examined. If 100 ml of sample is required the container should hold 125 ml.

A dechlorinating agent should be added to the bottle before it is sterilized. This can be accomplished by adding 0.1 ml of 10% solution of sodium thiosulphate per 100 ml of sample bottle capacity.

The sampling bottles sent out from the laboratory should be accompanied by a standard self-addressed mailing container with the report form and instructions for filling out the form and the proper technique for taking the sample.

B Sampling Procedure

When taking bacteriological samples keep the bottle stoppered until the sample is to to taken. When removing the stopper from the bottle be sure that the inside surface is not touched by the hands or comes into contact with anything but the water to be tested.

To avoid the loss of sodium thiosulfate in the sample bottles, do not rinse the bottle with the water being collected.

When taking the sample from a tap or pump, allow the water to run freely for 4-5 minutes before inserting the bottle into the water stream.

III SUBMISSION AND REPORTING

A As soon as the sample is taken all pertinent information asked for on the form accompanying the sterile bottle from the laboratory should be recorded.

B Submitting Sample

The sample should be submitted to the laboratory for examination by the most expeditious means of transport. In no case should the examination be started later than 24 hours after the sample is collected.

C Arrival at Laboratory

Upon arrival at the laboratory the sample should be logged in and assigned an identifying number. Such information to be logged in includes: source of sample, date and time of collection, temperature of sample source, name of collector, and date and time received at the laboratory.

D Laboratory Findings

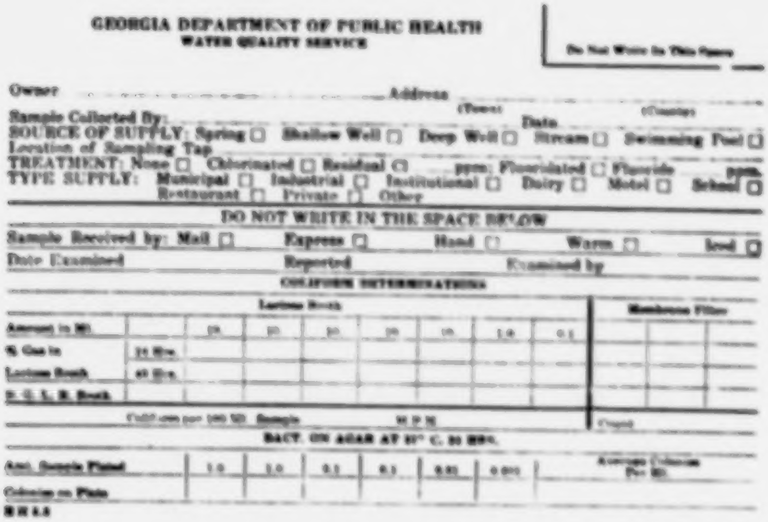
The laboratory report should contain such information as: date and time laboratory operations were started, sample volumes used for tests, the name of the laboratory worker. The laboratory form should also contain space for recording all results obtained, a summary of the results and quantitative determinations.

It would be well to have a more uniform system of laboratory reports throughout the United States. Information coming from the laboratories would be more meaningful to those responsible for the surveillance of water systems subject to Federal Quarantine Regulations.

REFERENCES

1 Public Health Service Drinking Water Standards 1962, U.S. Department of Health, Education, and Welfare.

2 Standard Methods for the Examination of Water and Waste Water 1965 - APHA, AWWA, WPCF.



GEORGIA DEPARTMENT OF PUBLIC HEALTH
WATER QUALITY SERVICE

Do Not Write In This Space

Owner ______ Address ______ (Town) ______ (County)

Sample Collected By: ______ Date ______

SOURCE OF SUPPLY: Spring ☐ Shallow Well ☐ Deep Well ☐ Stream ☐ Swimming Pool ☐

Location of Sampling Tap ______

TREATMENT: None ☐ Chlorinated ☐ Residual ☐ ______ ppm; Fluoridated ☐ Fluoride ______ ppm.

TYPE SUPPLY: Municipal ☐ Industrial ☐ Institutional ☐ Dairy ☐ Motel ☐ School ☐ Restaurant ☐ Private ☐ Other

DO NOT WRITE IN THE SPACE BELOW

Sample Received by: Mail ☐ Express ☐ Hand ☐ Warm ☐ Iced ☐

Date Examined ______ Reported ______ Examined by ______

COLIFORM DETERMINATIONS

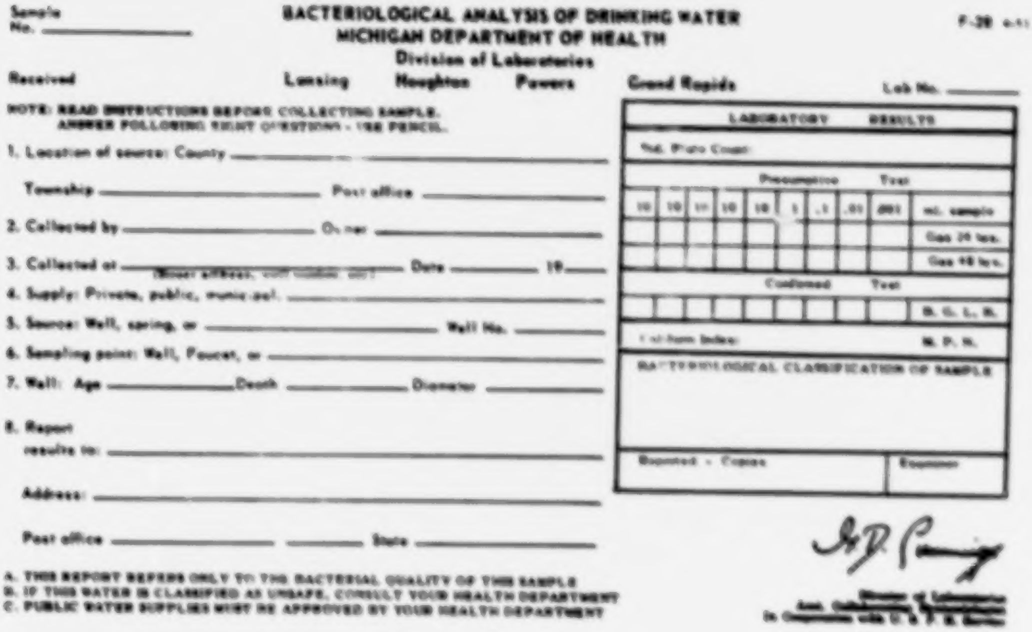
		Lactose Broth							Membrane Filter		
Amount in Ml.		10	10	10	10	10	1.0	0.1			
% Gas in	24 Hrs.										
Lactose Broth	48 Hrs.										
B. G. L. B. Broth											

Coliform per 100 Ml. Sample ______ M P N ______ Count ______

BACT. ON AGAR AT 37° C. 24 HRS.

Amt. Sample Plated	1.0	1.0	0.1	0.1	0.01	0.001	Average Colonies Per Ml.
Colonies on Plate							

BH 4.6



Sample No. ______

BACTERIOLOGICAL ANALYSIS OF DRINKING WATER
MICHIGAN DEPARTMENT OF HEALTH
Division of Laboratories

F-28 6-61

Received Lansing Houghton Powers Grand Rapids

Lab No. ______

NOTE: READ INSTRUCTIONS BEFORE COLLECTING SAMPLE.
ANSWER FOLLOWING EIGHT QUESTIONS - USE PENCIL.

1. Location of source: County ______
Township ______ Post office ______
2. Collected by ______ Owner ______
3. Collected at ______ (Street address, unit number, etc.) Date ______ 19___
4. Supply: Private, public, municipal. ______
5. Source: Well, spring, or ______ Well No. ______
6. Sampling point: Well, Faucet, or ______
7. Well: Age ______ Depth ______ Diameter ______
8. Report results to: ______
Address: ______
Post office ______ ______ State ______

LABORATORY RESULTS									
Std. Plate Count									
Presumptive Test									
10	10	10	10	10	1	.1	.01	.001	ml. sample
									Gas 24 hrs.
									Gas 48 hrs.
Confirmed Test									
									B. G. L. B.
Coliform Index									M. P. N.
BACTERIOLOGICAL CLASSIFICATION OF SAMPLE									
Reported - Copies									Examiner

A. THIS REPORT REFERS ONLY TO THE BACTERIAL QUALITY OF THIS SAMPLE
B. IF THIS WATER IS CLASSIFIED AS UNSAFE, CONSULT YOUR HEALTH DEPARTMENT
C. PUBLIC WATER SUPPLIES MUST BE APPROVED BY YOUR HEALTH DEPARTMENT

Director of Laboratories
[illegible]
In Cooperation with U. S. P. H. Service

MEDIA PREPARATION AND EQUIPMENT

I MATERIALS AND EQUIPMENT FOR MEDIA PREPARATION

Good laboratory technic and quality materials are essential in media preparation.

A Glassware

Borosilic glassware or other suitable noncorrosive equipment may be used. Utensils must be free from foreign residues, toxic or foreign materials.

B Distilled Water

Distilled or demineralized water which has been shown by proper tests to be free from toxic or inhibitory substances may be used.

C pH

Electrometric pH meters are recommended for the determination of pH of media. Color comparator blocks may be used except for media containing dyes such as brilliant green.

pH may be checked before and after sterilization and adjustments made as needed. Records should be maintained.

D Balance

Balance used for weighing media or media components should have a sensitivity of ± 2 grams for a 150 gram load.

E Autoclaves

Sterilization equipment should be equipped with proper valves and guages and at least one thermometer located in the exhaust line. The sterilizer should be of sufficient size to permit media to be loosely packed under normal loads. Operating capability of the sterilizer should be such that sterilizing temperature can be reached within 30 minutes from time the door is closed.

II CONCENTRATION OF MEDIA

A Basic formulas of all media described in Section IV are presented as single-strength media. Most media are used in the single-strength concentration.

B The concentration of primary inoculation media (media into which the measured portions of the original sample are delivered) requires special consideration.

1 When the amount of medium is 10 ml or greater, and the volume of sample or sample dilution is 1 ml or less, then single-strength medium is satisfactory.

2 When the sample volume introduced into the primary inoculation medium is greater than 1 ml, then it is necessary to compensate for the diluting effect of the sample on the culture medium. In such cases, it is necessary to increase the initial concentration of the medium so that after sample inoculation the concentration of nutrients in medium-plus-sample is equivalent to the concentration of nutrients in the single strength medium.

III PREPARATION OF MEDIA AND SOLUTIONS

A Lauryl Tryptose Broth (Lauryl Sulfate Broth)

1 Use: Primary inoculation medium in Presumptive Test

2 Composition:

Tryptose (or Trypticase or equivalent)	20.0	g
Lactose	5.0	g
Dibasic Potassium Phosphate (K_2HPO_4)	2.75	g
Monobasic Potassium Phosphate (KH_2PO_4)	2.75	g
Sodium Chloride	5.0	g
Sodium Lauryl Sulfate	0.1	g
(Total Dry Constituents	35.60	g)
Distilled Water	1000	ml

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the Environmental Control Administration and the Public Health Service

Sterilization: 12 - 15 minutes at 121°C
Reaction after sterilization: pH 6.8 (approximately)

3 Compensation for diluting effect of samples

No. ml medium in tube	Ml of sample or dilution	Nominal concentration before inoculation	No. grams dehyrated medium per liter
10	0.1-1.0	1x	35.6
10	10	2x	71.2
20	10	1.5x	53.4
35	100	4x	137.3

B Brilliant Green Lactose Bile Broth

1 Use: Confirmed Test

2 Composition

Peptone (Bacto or equivalent)	10.0	g
Lactose	10.0	g
Oxgall (dehydrated)	20.0	g
Brilliant Green	0.0133	g
(Total weight dry constituents	40.0133	g)
Distilled Water	1000	ml

Sterilization: 12 - 15 minutes at 121°C
Reaction after sterilization: pH 7.1 to 7.4

C Eosin Methylene Blue Agar, Levine

1 Use: Isolation of coliform-like colonies as a preliminary to Completed Test procedures.

2 Composition

Peptone (Bacto or equivalent)	10	g
Lactose	10	g
Dipotassium Phosphate (K_2HPO_4)	2	g
Agar	20	g
Eosin Y	0.4	g
Methylene Blue	0.65	g
(Total weight dry constituents	43.05	g)
Distilled water	1000	ml

Sterilization: 12 - 15 minutes at 121°C

3 Special suggestions on preparation:

a This medium can be prepared and dispensed into bottles or flasks in portions of 100 ml or 200 ml each. The medium may be stored for extended periods in cool places out of the light.

b When ready for use of such medium, the medium should be melted by immersion of the bottle of prepared medium in a boiling water bath, after which it is dispensed into sterile Petri dishes in portions of approximately 15 ml. After cooling and solidifying in the Petri dish, the medium is ready for use. It should be used preferably on the day it is poured into Petri dishes, but can be stored for a day or two in the refrigerator.

c An alternate method of preparing this medium requires preparation of an agar base medium (see 2, above) which includes all the constituents of the medium except the dyes. When ready to use such a preparation, the agar base medium is melted in a water bath, and to each 100 ml of the melted agar base medium, 2 ml of 2% of aqueous solution of eosin Y and 1.3 ml of 0.5% methylene blue solution is delivered with a pipet. The medium is mixed thoroughly, poured into Petri dishes, and used as previously described.

D Agar Slants

1 Use: This medium is used in the Confirmed Test, to cultivate pure cultures of strains of bacteria being cultivated in preparation of a Gram-stained smear.

2 Composition: The medium is nutrient agar

Peptone	5.0	g
Beef extract	3.0	g
Agar	15.0	g
(Total weight dry constituents:	23.0	g)
Distilled water	1000	ml

Sterilization: 15 minutes at 121°C

Reaction after sterilization: pH 6.8 approximately

3 Special instructions: Dissolve the constituents, using heat as needed; dispense in amounts of approximately 8 ml per tube. Screw-capped tubes extend shelf life of the medium. After sterilization, remove the melted medium from the autoclave and place in a slanting position until the medium has become solidified. A routine procedure should be established so that a uniform volume of medium and a uniform surface of slanted medium be present in each tube. While this has no particular bearing on Standard Methods procedures, certain other laboratory procedures do require uniform exposed surface area of the slanted medium.

E Plate Count Agar

1 Use: In this course, the medium is used in the distilled water test described in Outline 34. It is not used in other Standard Methods procedures described in this course manual.

2 Composition: (Tryptone Glucose Yeast Agar)

Pepton-tryptone (or equivalent)	5.0	g
Yeast extract	2.5	g
Glucose (dextrose)	1.0	g
Agar	15.0	g

(Total weight dry constituents 23.5 g)

Distilled water 1000 ml

Sterilization: 15 minutes at 121°C

Reaction after sterilization: pH 7.0 ± 0.1

3 Special instructions in preparation: Use heat as needed to dissolve and melt the constituents. Dispense the medium in flasks or bottles in portions of 100 or 200 ml each. In this state it can be preserved for many months, provided that is is protected from evaporation of the water.

When ready to use, melt the medium by heating, and cool to 45°C.
At this temperature the medium still should be melted, and will be satisfactory for preparation of pour plates for plate counts.

F EC Broth

1 Use: Test for fecal coliform bacteria

2 Composition:

Tryptose (Bacto or equivalent)	20.0	g
Lactose	5.0	g
Bile Salts (Bacto #3 or equivalent)	1.5	g
Dipotassium phosphate (K_2HPO_4)	4.0	g
Monopotassium phosphate (KH_2PO_4)	1.5	g
Sodium chloride	5.0	g

(Total weight dry constituents 37.0 g)

Distilled water 1000 ml

Sterilization: 12 - 15 minutes at 121°C

Reaction after sterilization: pH 6.9

3 This medium is dispensed into culture tubes with inverted fermentation vials and metal caps.

G Azide Dextrose Broth

1 Use: Primary inoculation medium for fecal streptococcal presumptive test.

2 Composition:

Beef extract	4.5	g
Tryptone or Polypeptone	15.	g
Glucose	7.5	g
Sodium chloride	7.5	g
Sodium azide	0.2	g

(Total dry constituents 34.7 g)

Distilled water 1000 ml

Sterilization: 12 - 15 minutes at 121° C

Reaction after sterilization: about pH 7.2

3 Fermentation vials are not used with azide dextrose broth.

H Ethyl violet Azide Broth

1 Use: Confirmed test for fecal streptococci

2 Composition:

Tryptone or Biosate	20 g
Glucose	5 g
Sodium chloride	5 g
Potassium phosphate, diabasic (K_2HPO_4)	2.7g
Potassium phosphate, monobasic (KH_2PO_4)	2.7g
Sodium azide	0.4g
Ethyl violet (certified dye if available)	.00083g
(Total dry constituents	35.8g)
Distilled water	1000 ml

Sterilization: 12 - 15 minutes at 121°C

Reaction after sterilization: about pH 7

I Buffered Dilution Water

1 Use: Preparation of sample dilutions preliminary to primary inoculation, in membrane filter work, and in plate counts.

2 Composition

a Stock phosphate buffer solution

Monobasic Potassium Phosphate (KH_2PO_4)	34.0 g
Distilled Water	500 ml

1N NaOH solution (about 175 ml) to give pH 7.2

Distilled water sufficient to bring final volume to 1000 ml

b Working solution of phosphate buffered distilled water

Stock phosphate buffer solution	1.25 ml
Distilled water	1000 ml

3 Preparation and handling:

a Stock solution: After preparation the stock solution should be stored in the refrigerator until use. If at any time evidence of mold or other contamination appears, the stock solution should be discarded and a fresh solution prepared.

b Working solution: dispense the required amount into distilled water, and deliver into screw-capped bottles for dilution water. The amount added should be such that, after sterilization, the bottles will contain 99 + 2 ml of the dilution water. Ordinarily this requires initial addition of approximately 102 ml of the solution prior to sterilization.

c Sterilization is 20 minutes at 121°C.

d Tightly stoppered bottles of the dilution water, protected against evaporation, in suitable containers, appear to last indefinitely.

J Solutions for Gram Stain

1 Ammonium oxalate crystal violet solution:

a Dissolve 2 g crystal violet (approximately 85% dye content) in 20 ml of 95% ethyl alcohol.

b Dissolve 0.8 grams ammonium oxalate in 80 ml distilled water.

c Mix solutions a and b.

d Filter through cheesecloth or coarse filter paper.

e Problems with the gram stain technique frequently are traceable to the ammonium oxalate crystal violet solution. In the event that decolorization does not seem satisfactory, the amount of crystal violet in the solution can be reduced to as little as 10% of the recommended amount.

2 Lugol's iodine: dissolve 1 g iodine crystals and 2 g potassium iodide in the least amount (usually about 5 ml) of distilled water in which they are soluble. After all crystals are in solution, add sufficient distilled water to bring the final solution to a volume of 300 ml.

3 Counterstain: Dissolve 2.5 grams of safranin in 100 ml of 95% ethyl alcohol. For the working solution of counterstain, add 10 ml of this solution of safranin to 100 ml of distilled water.

IV OTHER LABORATORY EQUIPMENT

A Pipets

Pipets should deliver accurately and quickly the required amount, with the error of calibration not exceeding 2.5%. Pipets conforming to the lastest edition of Standard Methods for the Examination of Dairy Products may be used.

B Pipet Boxes

Pipet boxes of aluminium or stainless steel are recommended. Wrapping paper may be substituted. Copper pipet boxes are prohibited.

C Dilution bottles of tubes.

Dilution bottles or tubes should be of resistant glass, preferably borosilicate, closed with glass stoppers or screw caps equipped with non toxic liners. Non toxic plastic bottles may also be used.

D Petri Dishes

Plastic or glass petri dishes may be used. Copper Sterilization boxes for petri dishes should not be used.

E Incubators

Incubators must maintain a constant temperature at all times. Water jacketed, anhydrous types and walk-in incubators should have low temperature heating units and be properly insulated. An NBS accurate thermometer checked by an NBS certified thermometer or equivalent, with the bulb immersed in liquid should be maintained on each shelf. Forced air systems are desirable. Temperatures should not vary more than 0.5°C for 35° incubation or 0.2°C for a fecal coliform waterbath incubator. Forced circulation is desirable in waterbath incubators.

F Hot Air Sterilizing Ovens

Hot air sterilizing ovens should be capable of maintaining sterilizing temperatures and be of sufficient size to accomodate effective work load. These ovens should be equipped with a thermometer capable of registering accurate temperatures in the range of 160°-180° C.

REFERENCES

1 Standard Methods for the Examination of Water and Wastewater 12 Ed. 1965.

2 Media and Solutions for Multiple Dilution Tube Methods, Harold L. Jeter, "Current Practices in Water Microbiology", Training Manual, FWPCA.

THE MEMBRANE FILTER IN WATER BACTERIOLOGY

I HISTORICAL BACKGROUND

There is sometimes a tendency to look upon membrane filters and their bacteriological applications as new developments. Both the filters and many of their present bacteriological applications are derived from earlier work in Europe.

A Some European developments prior to 1947 are as follows:

1 Fick is credited with application of collodion membranes in biological investigations in 1855.

2 Sanarelli, in 1891, reported development of membrane filters impermeable to bacteria but permeable to their toxins.

3 Bechhold, in the early 1900's made a systematic study of the physico-chemical properties of a number of varieties of these membranes. After 1911 numerous investigations were made in several countries with respect to the properties of collodion membranes.

4 Zsigmondy and Bachmann, 1916-1918, developed improved production methods which were applicable on a commercial scale. Membrane filters have been produced for many years at the Membranfiltergesellschaft, Sartorious Werke, in Goettingen, Germany. In 1919 Zsigmondy applied for a U.S. patent on his production methods; it was granted in 1922.

5 In the 1930's, W. J. Elford in England, and P. Graber in France, made new contributions in developing and teaching methods for making collodion membranes with controlled pore size.

6 Before World War II filtration procedures using the Zsigmondy membrane had been suggested for the determination of bacterial counts, coliform determinations, and isolation of pathogenic bacteria from water and other fluids. Most early interest in developing these techniques seems to have been in Germany and in Russia. During World War II Dr. G. Mueller applied membrane filter techniques to the bacteriological examination of water, following bomb destruction of many of the laboratories.

B Developments in the United States

1 In 1947, Dr. A. Goetz reported on a mission to Germany as a scientific consultant to the Technical Industrial Intelligence Branch, U. S. Department of Commerce. He obtained detailed information about the nature, method of preparation, and specific bacteriological applications of the Zsigmondy Membranfilter being manufactured by the Membranfiltergesellschaft in Goettingen.

2 After his return to this country, Dr. Goetz developed methods for preparing and improved type of membrane filter from domestic materials. On a small scale he manufactured filters under a government contract; afterward membrane filter manufacture was continued by a commercial organization

3 In 1950, bacteriologists of the Public Health Service began intensive study of the applications of membrane filters in bacteriological examinations of water. Their first report was published in 1951, and was followed by numerous reports of other similar investigations. Such studies have been widely expanded, as indicated in references shown elsewhere in this manual.

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the FWPCA and the U.S. Department of the Interior.

4 In 1955 the 10th Edition of "Standard Methods for the Examination of Water, Sewage, and Industrial Wastes" included a tentative method for coliforms by membrane filter method. In the 11th and 12th editions, the membrane filter method for coliforms has become official. In addition, methods for enterococcus (fecal streptococci) are included as tentative methods.

5 The membrane filter is an official method for examination of potable waters in interstate commerce. The Public Health Service Drinking Water Standards (1962) state "Organisms of the coliform group... All the details of technique ...shall be in accordance with Standard Methods for Examination of Water and Wastewater, current edition..." Thus, acceptance by Standard Methods as official automatically validates a method for use with interstate waters.

II PROPERTIES OF MEMBRANE FILTERS

Membrane filters used in water bacteriology are flat, highly porous, flexible plastic discs about 0.15 millimeters in thickness and usually 47-50 millimeters in diameter.

A Principle of Manufacture

The procedures described below are from FIAT Report 1312. While the methods indicated by Goetz do not necessarily describe the current manufacturing processes, it is assumed that similar principles of manufacture still apply.

1 One or more cellulose esters, such as cellulose nitrate, is dissolved in a suitable solvent.

2 Water, or some other liquid insoluble in the cellulose solution, is added and mixed, to form an emulsion having great uniformity in size and distribution of droplets of the insoluble liquid.

3 The emulsion is cast on plates and dried in an environment rigidly controlled as to humidity and temperature. The droplets of insoluble fluid retain their size and identity in the dried film, eventually becoming the pores of the finished membrane.

4 The dried porous film is cut into filter discs of the desired size. Representative discs are subjected to control tests for accurate determination of the pore size obtained.

B Some Important Characteristics of Membrane Filters

1 Membrane filters are flat, and have extremely smooth surfaces.

2 The pore structure occupies most of the volume of a filter. This permits rapid filtration of aqueous suspensions.

a With Millipore Type HA filters, 67 ± 10 ml of distilled water will pass through each square centimeter per minute with a differential pressure of 70 cm. Hg., at 25°C.

b The filtration rate of filters from Carl Schleicher and Schuell Company are designated in terms of the number of milliliters per minute per square cm. at 700 mm Hg differential pressure. The B-9 Type filter with black grid has a flow rate of 65 milliliters per minute per sq. cm.

c Oxoid membrane filters filter water through at the rate of 80 ml per minute per square centimeter at 70 cm. Hg. differential pressure at 20°C.

3 Particle retention by membrane filters is at or very near the filter surface, by a mechanical, sieve-like action. (This applies to hydrosols, not to aerosols.) Through manufacturing control it is possible to make membrane filters with controlled pore size, within narrow limits.

a Millipore filters are available in 10 designated pore sizes, ranging from 10 mμ to 8 μ. For most water bacteriological tests, Millipore Type HA filters, white, grid-marked, 47 mm in diameter, are recommended. These filters have pores with diameter 0.45 ± 0.02 μ.

b Membrane filters from Carl Schleicher and Schuell Company are available in pore sizes ranging from 5 millimicron to 8 micron. The B-9 type has an average purosity of 0.45 micron. S & S filters are available in 23 various diameters. Diameters of 25, 27, 47 and 50 millimeter are most commonly used.

c Oxoid cellulose acetate membrane filters are apparently sold only in the pore size for bacteriological analysis, 0.5 - 1.0 μ. These filters are described as being 0.005 inches thick and very porous. One surface is a thin layer of very fine pores the size of which varies from 0.5 to 1.0 μ, while the remainder of the filter consist of pore sizes 3 - 5.

4 Membrane filters are wettable. Thus, after sample filtration, when a filter is placed on moist culture medium the medium diffuses through the pores and is available to organisms collected on the opposite surface.

5 Membrane filters are free of soluble chemical substances inhibitory to bacterial growth. Water soluble plasticizers are included in one commercially produced filter (glycerol, 2.5%). The cellulose esters themselves have some absorbing tendency illustrated by some dyes and heavy metals. Total ash is very low, less than 0.0001%.

6 Membrane filters have a uniform index of refraction. With membrane filters, this index is N_d: 1.5. When wetted with a liquid having refractive index within this range, the filters become transparent. This property permits direct microscopic examination of particulate matter collected on the filter surface.

7 Temperature resistance depends on plastics used in the filter. The nitrocellulose membrane filter is stable dry up to 125°C in air. Gelman membranes of cellulose triacetate are advertised to withstand dry heat to 266°C. In general, however, membranes in current use must be sterilized cautiously. Consult the laboratory equipment discussion for details. Overheating of all types interferes with filtration by blocking pores.

C Nomenclature

Membrane filters used in bacteriological tests on water are known under several names. Though the names are different, the filters are similar in form, properties, and method of use. Names commonly encountered are:

1 Membrane filter. This is the general name for filters made according to the general principles and having the properties discussed above. The term "membrane filter" is most used in technical reports on filters of this type.

2 Molecular filter. This name used by Goetz for the improved type of filter that he and his associates developed after study of the manufacturing methods at the Membranfiltergesellschaft in Goettingen, Germany.

3 Millipore filter is a trade name for membrane filters made by the Millipore Filter Corporation.

4 Bac-T-Flex filter is a trade name applied to certain membrane filters made by Carl Schleicher and Schuell Company.

5 Oxoid filter is a trade name applied to filters made by Oxo, Ltd., London, England.

6 Micropore, Polypore and Metricel have been trade names used by the Gelman Instrument Company.

III APPLICATIONS IN WATER BACTERIOLOGY

A The basic cultural procedures for bacteriological tests on membrane filters are:

1 A sample is filtered through a membrane filter.

2 The filter is placed in a culture container, on an agar medium or a paper pad impregnated with moist culture medium.

3 The inoculated filter is incubated under prescribed conditions of time, temperature, and humidity.

4 After incubation, the resulting culture is examined and necessary interpretations and/or additional tests are made.

B With variations in such factors as culture media, incubation time, and combinations with other cultural and biochemical tests, several different kinds of tests are available.

1 Total bacterial counts are made by cultivation of bacteria on membrane filters using an enriched all-purpose culture medium.

2 Tests for bacterial indicators of pollution.

a Coliform tests

1) The direct membrane filter tests for coliforms is one in which, after sample filtration, the membrane filter is incubated in contact with one or more special media. At least one of the media is a selective, differential medium including components which permit coliform bacteria to develop colonies easily recognizable by form, color, sheen, or other characteristics.

2) A verified membrane filter coliform test can be used when needed as a supplement to the direct membrane filter test. Pure cultures are obtained from individual colonies differentiated on the membrane filter and subjected to further cultural, biochemical, and staining tests to establish the identity of the colonies being studied.

3) The delayed membrane filter coliform test was developed to overcome bacterial changes frequently occurring when there is a delay of one to several days between sample collection and the initiation of laboratory tests. The test consists of sample filtration at or shortly after the time of sample collection. The inoculated filter is placed on a preservative medium and taken or sent to a laboratory, where it is transferred to a growth medium for the differentiation of coliform colonies. After incubation the culture is examined and the results are evaluated as for the direct membrane filter coliform test.

4) A new medium and technique for detecting and counting fecal coliform bacteria has been developed by SEC research personnel. This new medium currently is being used increasingly in water pollution studies.

b Selective, differential, culture media have been developed for direct cultural tests for members of the enterococcus group of bacteria.

3 Tests for pathogenic bacteria

a Workers are currently testing new media for the differentiation of members of the Salmonella-Shigella group of enteric pathogens. Available information indicates potential usefulness of a screening medium for differentiation of nonlactose-fermenting, non urease-producing bacteria.

b One medium has been found useful for screening tests in detection of Salmonella typhosa.

c Further confirmatory cultural, biochemical, and serological tests are necessary to establish the identity of bacteria differentiated with these screening media.

C Membrane filter techniques can be applied both in the laboratory and under field conditions. Several varieties of portable membrane filter field units have been developed on a commercial basis.

IV ADVANTAGES AND LIMITATIONS

This evaluation is limited to tests for the coliform group. Similar, but separate evaluations would have to be made for any other bacteriological test.

A Advantages

1 Results are obtained in approximately 24 hours, as compared with 48-96 hours required for the standard fermentation tube method.

2 Much larger, and hence more representative samples of water can be sampled routinely with membrane filters.

3 Numerical results from membrane filters have much greater precision (reproducibility) than is expected with the fermentation tube method.

4 The equipment and supplies required are not bulky. A great many samples can be examined with minimum requirements for laboratory space, equipment, and supplies.

B Limitations

1 Samples having high numbers of noncoliform bacteria capable of growing on Endo type culture media sometimes give difficulty. In such cases a high ratio of these noncoliform bacteria to coliforms results in poor sheen production, or even suppression, of the coliform organisms.

2 In samples having low coliform counts and relatively great amounts of suspended solids, bacterial growth sometimes develops in a continuous film on the membrane surface. In such cases the typical coliform sheen sometimes fails to develop.

3 Some samples containing as much as 1 milligram per liter of copper or zinc, or both, show irregular coliform bacterial results.

4 Occasional strains of bacteria growing on membrane filters producing sheen colonies prove, on subsequent testing, to be acid but not gas-producers from lactose. Where this occurs it may give a falsely-high indication of coliform density.

Such limitations as these are not frequent, but they do occur often enough to require consideration. In samples where these difficulties often occur, the best course of action often is to avoid use of membrane filter methods and use the multiple fermentation tube procedures.

V SUMMARY

The development of membrane filters and their bacterial applications has been discussed briefly, from their European origin to their current status in this country. Membrane filters currently available here have been described, and their properties have been considered. Applications of membrane filters in water bacteriology are indicated in general terms. Some of the advantages and limitations of membrane filter methods are presented for coliform tests.

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EXAMINATION OF WATER FOR COLIFORM AND FECAL STREPTOCOCCUS GROUPS
(Multiple Dilution Tube (MPN) Methods)

I INTRODUCTION

The subject matter of this outline is contained in three parts, according to the following plan:

A Part 1

1 Fundamental aspects of multiple dilution tube ("most probable numbers") tests, both from a qualitative and a quantitative viewpoint.

2 Laboratory bench records.

3 Useful techniques in multiple dilution tube methods.

4 Standard supplies, equipment, and media in multiple dilution tube tests.

B Part 2

Detailed, day-by-day, procedures in tests for the coliform group and subgroups within the coliform group.

C Part 3

Detailed, day-by-day, procedures in tests for members of the fecal streptococci.

II BASIS OF MULTIPLE TUBE TESTS

A Qualitative Aspects

1 For purely qualitative aspects of testing for indicator organisms, it is convenient to consider the tests applied to one sample portion, inoculated into a tube of culture medium, and the follow-up examinations and tests on results of the original inoculation. Results of testing procedures are definite: positive (presence of the organism-group is demonstrated) or negative (presence of the organism-group is not demonstrated).

2 Test procedures are based on certain fundamental assumptions:

a First, even if only one living cell of the test organism is present in the sample, it will be able to grow when introduced into the primary inoculation medium;

b Second, growth of the test organism in the culture medium will produce a result which indicates presence of the test organism; and,

c Third, extraneous organisms will not grow, or if they do grow, they will not limit growth of the test organism; nor will they produce growth effects that will be confused with those of the bacterial group for which the test is designed.

3 Meeting these assumptions usually makes it necessary to conduct the tests in a series of stages (for example, the Presumptive, Confirmed, and Completed Test stages, respectively, of standard tests for the coliform group).

4 Features of a full, multi-stage test

a First stage: The culture medium usually serves primarily as an enrichment medium for the group tested. A good first-stage growth medium should support growth of all the living cells of the group tested, and it should include provision for indicating the presence of the test organism being studied. A first-stage medium may include some component which inhibits growth of extraneous bacteria, but this feature never should be included if it also inhibits growth of any cells of the group for which the test is designed. The Presumptive Test for the coliform group is a good

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NOTE: Mention of commercial products and manufacturers does not imply endorsement by the Federal Water Pollution Control Administration and the U.S. Department of the Interior.

example. The medium supports growth, presumably, of all living cells of the coliform group; the culture container has a fermentation vial for demonstration of gas production resulting from lactose fermentation by coliform bacteria, if present; and sodium lauryl sulfate may be included in one of the approved media for suppression of growth of certain noncoliform bacteria. This additive apparently has no adverse effect on growth of members of the coliform group in the concentration used. If the result of the first-stage test is negative, the study of the culture is terminated, and the result is recorded as a negative test. No further study is made of negative tests. If the result of the first-stage test is positive, the culture may be subjected to further study to verify the findings of the first stage.

b Second stage: A transfer is made from positive cultures of the first-stage test to a second culture medium. This test stage emphasizes provision to reduce confusion of results due to growth effects of extraneous bacteria, commonly achieved by addition of selective inhibitory agents. (The Confirmed Test for coliforms meets these requirements. Lactose and fermentation vials are provided for demonstration of coliforms in the medium. Brilliant green dye and bile salts are included as inhibitory agents which tend to suppress growth of practically all kinds of noncoliform bacteria, but do not suppress growth of coliform bacteria when used as directed).

If result of the second-stage test is negative, the study of the culture is terminated, and the result is recorded as a negative test. A negative test here means that the positive results of the first-stage test were "false positive," due to one or more kinds of extraneous bacteria. A positive second-stage test is partial verification of the positive results obtained in the first-stage test; the culture may be subjected to final verification through application of still further testing procedures. In routine practice, most sample examinations are terminated at the end of the second stage, on the assumption that the result would be positive if carried to the third, final verification stage. This practice should be followed only if adequate testing is done to demonstrate that the assumption is valid. Some workers recommend continuing at least 5% of all sample examinations to the third stage to demonstrate the reliability of the second-stage results.

B Quantitative Aspects of Tests

1 These methods for determining bacterial numbers are based on the assumption that the bacteria can be separated from one another (by shaking or other means) resulting in a suspension of individual bacterial cells, uniformly distributed through the original sample when the primary inoculation is made.

2 Multiple dilution tube tests for quantitative determinations apply a Most Probable Number (MPN) technique. In this procedure one or more measured portions of each of a stipulated series of decreasing sample volumes is inoculated into the first-stage culture medium. Through decreasing the sample increments, eventually a volume is reached where only one cell is introduced into some tubes, and no cells are introduced into other tubes. Each of the several tubes of sample-inoculated first-stage medium is tested independently, according to the principles described above, in the qualitative aspects of testing procedures.

3 The combination of positive and negative results is used in an application of probability mathematics to secure a single MPN value for the sample.

4 To obtain MPN values, the following conditions must be met:

a The testing procedure must result in one or more tubes in which the test organism is demonstrated to be present; and

b The testing procedure must result in one or more tubes in which the test organism is not demonstrated to be present.

5 The MPN value for a given sample is obtained through the use of MPN Tables. The use of such tables is discussed in Outline 21 of this course manual. It is emphasized that the precision of an individual MPN value is not great when compared with most physical or chemical determinations. The effect on precision of results, based on numbers of tubes inoculated per sample volume, is shown in Table 1.

6 Standard practice in water pollution surveys conducted by this organization, is to plant five tubes in each of series of sample increments, in sample volumes decreasing at decimal intervals. For example, in testing known polluted waters, the initial sample inoculations might consist of 5 tubes each in volumes of 0.1, 0.01, 0.001, and 0.0001 ml, respectively.

III LABORATORY BENCH RECORDS

A Features of a Good Bench Record Sheet

1 Provides complete identification of the sample.

2 Provides for full, day-by-day information about all tests performed on the sample.

3 Provides easy step-by-step record applicable to any portion of the sample.

4 Provides for recording of quantitative result which will be transcribed to subsequent reports.

5 Minimizes the amount of writing by the analyst.

B There is no such thing as "standard" bench sheet for multiple tube tests; there are many versions of bench sheets. Some are prescribed by administrative authority (such as the Office of a State Sanitary Engineer); others are devised by laboratory or project personnel to meet specific needs. It is not the purpose of this discussion to recommend an "ideal" bench form; however, the form used in this training course manual is essentially similar to that used in certain research laboratories of this

Table 1. Approximate Confidence Limits for Bacterial Densities as Per Cent of MPN as Determined from Various Numbers of Tubes in Three Decimal Dilutions*

Number of tubes in each dilution	50%		75%		80%		90%		95%	
	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper
1	33	186	18	340	15	402	10	637	6.5	955
2	47	160	31	246	27	276	20	383	15	511
3	53	150	38	215	34	237	26	311	21	395
5	64	139	49	182	46	196	37	241	31	289
10	76	127	63	152	60	160	52	184	46	208

*The interpretation of these figures is as follows: When MPN estimates are made on the basis of dilution tests using one tube in each of three decimal dilutions, you will be right 50% of the time if you say that the true bacterial density is between 33% and 186% of the MPN. If you had used 5 tubes in each dilution you could reduce this interval to from 64% to 139% of the MPN and still be right 50% of the time. If a greater certainty were desired, say 95%, you would have to widen this interval to from 31% to 289%.

BACTERIOLOGY BENCH SHEET

Multiple Dilution Tube Tests

Project Ohio River Survey

Sample Station Broadway Landing

Collection Data

Date 2/16/69 Time 8:50 By KJ

Temperature 8 °C pH 7.3

Other Observations

Analytical Record

Bench Number of Sample 2

Analyst Zobel-Jeter

Test started at 11:45 By HLJ

ml sample	Coliform Test								Fecal coli-form	Fecal Streptococcus				Remarks
	LTB		BGLB		EMB	LSTB		Gram stain		A - D		EVA		
	24	48	24	48	24	24	48		24	24	48	24	48	
10														
1.0														
0.1														
0.01														
0.001														
0.001														

Coliform MPN/100 ml

Confirmed:

Completed:

Fecal Coliform MPN:

Fecal Streptococcus MPN/100 ml

A-D - EVA:

Figure 1. SAMPLE BENCH SHEET

organization. The student enrolled in the course for which this manual is written should make himself thoroughly familiar with the bench sheet and its proper use. See Figure 1.

IV NOTES ABOUT WORKING PROCEDURES IN THE LABORATORY

A Each bacteriological examination of water by multiple dilution tube methods requires a considerable amount of manipulation; much of this is quite repetitious. Laboratory workers must develop and maintain good routine working habits, with constant alertness to guard against lapses into careless, slip-shod laboratory procedures and "short cuts" which only can lead to lowered quality of laboratory work.

The student reader is urged to review the form for laboratory surveys (PHS-875, Rev. 1966) used by Public Health Service personnel charged with responsibility for accreditation of laboratories for examination of water under Interstate Quarantine regulations.

B Specific attention is brought to the following, by no means exhaustive, critical laboratory procedures in multiple dilution tube tests:

1 Original sample

a Follow prescribed care and handling procedures before testing.

b Maintain absolute identification of sample at all stages in testing.

c Vigorously shake samples (and sample dilutions) before planting in culture media.

2 Sample measurement into primary culture medium

a Sample portions must be measured accurately into the culture medium for reliable quantitative tests to be made. Standard Methods prescribes that calibration errors should not exceed ± 2.5%.

Table 2. SUGGESTED LABELING SCHEME FOR ORIGINAL CULTURES AND SUBCULTURES IN MULTIPLE DILUTION TUBE TESTS

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Sample volume represented
Bench number Volume & tube	312 A	312 B	312 C	312 D	312 E	Tubes with 10 ml of sample
Bench number Volume & tube	312 a	312 b	312 c	312 d	312 e	Tubes with 1 ml of sample
Bench number Volume & tube	312 $\underline{a}$	312 $\underline{b}$	312 $\underline{c}$	312 $\underline{d}$	312 $\underline{e}$	Tubes with 0.1 ml of sample
Bench number Volume & tube	312 1a	312 1b	312 1c	312 1d	312 1e	Tubes with 0.01 ml of sample
Bench number Volume & tube	312 2a	312 2b	312 2c	312 2d	312 2e	Tubes with 0.001 ml of sample

The labeling of cultures can be reduced by labeling only the first tube of each series of identical sample volumes in the initial planting of the sample. All subcultures from initial plantings should be labeled completely.

Suggested sample measuring practices are as follows: Mohr measuring pipets are recommended. 10 ml samples are delivered at the top of the culture tube, using 10 ml pipets. 1.0 ml samples are delivered down into the culture tube, near the surface of the medium, and "touched off" at the side of the tube when the desired amount of sample has been delivered. 1.0 ml or 2.0 ml pipets are used for measurement of this volume. 0.1 ml samples are delivered in the same manner as 1.0 ml samples, using great care that the sample actually gets into the culture medium. Only 1.0 ml pipets are used for this sample volume. After delivery of all sample increments into the culture tubes, the entire rack of culture tubes may be shaken gently to carry down any of the sample adhearing to the wall of the tube above the medium.

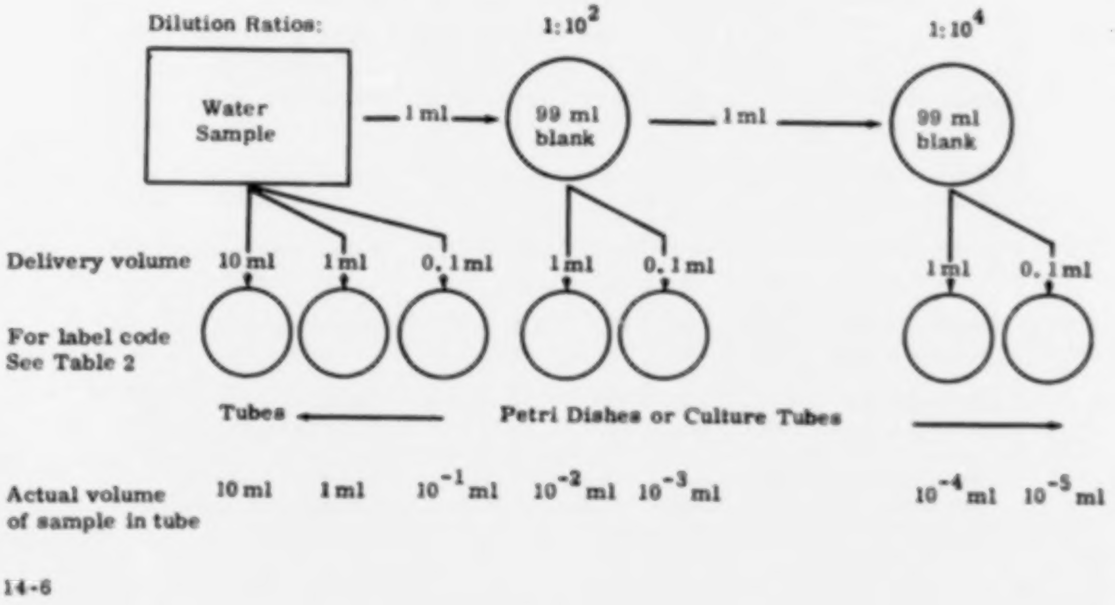
Workers should demonstrate by actual tests that the pipets and the technique in use actually delivers the rated volumes within the prescribed limits of error.

b Volumes as small as 0.1 ml routinely can be delivered directly from the sample with suitable pipets. Lesser sample volumes first should be diluted, with subsequent delivery of suitable volumes of diluted sample into the culture medium. A diagrammatic scheme for making dilutions is shown on Figure 2.

3 Reading of culture tubes for gas production

a On removal from the incubator, shake culture rack gently, to encourage release of gas which may be super saturated in the culture medium.

Figure 2. PREPARATION OF DILUTIONS


Dilution Ratios:
1:10^2
1:10^4
Water Sample
1 ml
99 ml blank
1 ml
99 ml blank
Delivery volume
10 ml
1 ml
0.1 ml
1 ml
0.1 ml
1 ml
0.1 ml
For label code See Table 2
Tubes
Petri Dishes or Culture Tubes
Actual volume of sample in tube
10 ml
1 ml
10^-1 ml
10^-2 ml
10^-3 ml
10^-4 ml
10^-5 ml

b Gas in any quantity is a positive test. It is necessary to work in conditions of suitable lighting for easy recognition of the extremely small amounts of gas inside the tops of some fermentation vials.

4 Reading of liquid culture tubes for growth as indication of a positive test requires good lighting. Growth is recognized by any amount of increased turbidity or opalescence in the culture medium, with or without deposit of sediment at the bottom of the tube.

5 Transfer of cultures with inoculation loops and needles

a Always sterilize inoculation loops and needles in flame immediately before transfer of culture; do not lay it down or touch it to any nonsterile object before making the transfer.

b After sterilization, allow sufficient time for cooling, in the air, to avoid heat-killing bacterial cells on the hot wire.

c Loops should be 3 mm in inside diameter, with a capability of holding a drop of water or culture.

For routine standard transfers requiring transfers of 3 loopsful of culture, many workers form three 3-mm loops on the same length of wire.

6 As an alternative to use of standard inoculation loops, increasing attention is being given to use of sterilized applicator sticks.

The applicator sticks are dry heat sterilized (autoclave sterilization is not acceptable because of possible release of phenols if the wood is steamed.) and are used on a single-service basis. Thus, for every culture tube transferred, a new applicator stick is used.

This use of applicator sticks is particularly attractive in field situations where it is inconvenient or impossible to provide a gas burner suitable for sterilization of the inoculation loop. In addition, use of applicator sticks is favored in laboratories where room temperatures are significantly elevated by use of gas burners.

It is anticipated that use of wooden applicator sticks in culture transfers will receive formal sanction in the next (13th) edition of Standard Methods.

7 Streaking cultures on agar surfaces

a All streak-inoculations should be made without breaking the surface of the agar. Learn to use a light touch with the needle; however, many inoculation needles are so sharp that they are virtually useless in this respect. When the needle is platinum or platinum-iridium wire, it sometimes is beneficial to fuse the working tip into a small sphere. This can be done by momentary insertion of a well-insulated (against electricity) wire into a carbon arc, or some other extremely hot environment. The sphere should not be more than twice the diameter of the wire from which it is formed, otherwise it will be entirely too heat-retentive to be useful.

When the needle is nichrome resistance wire, it cannot be heat-fused; the writer prefers to bend the terminal 1/16 - 1/8" of the wire at a slight angle to the overall axis of the needle. The side of the terminal bent portion of the needle then is used for inoculation of agar surfaces.

b When streaking for colony isolation, avoid using too much inoculum. The streaking pattern is somewhat variable according to individual preference. The procedure favored by the writer is shown in the accompanying figures. Note particularly that when going from any one stage of the streaking to the next, the inoculation needle is heat-sterilized.

8 Preparation of cultures for Gram stain

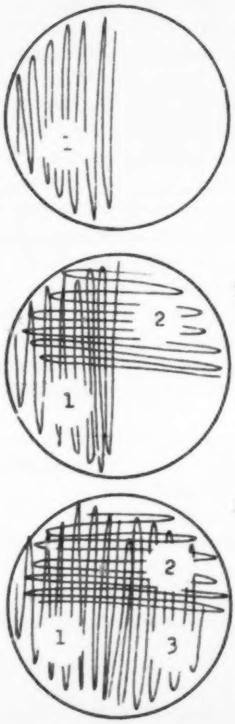
a The Gram stain always should be made from a culture grown on a nutrient agar surface (nutrient agar slants are used here) or from nutrient broth.

b The culture should be young, and should be actively growing. Many workers doubt the validity of the Gram stain made on a culture more than 24 hours old.

c Prepare a thin smear for the staining procedure. Most beginning workers tend to use too much bacterial suspension in preparing the dried smear for staining. The amount of bacteria should be so small that the dried film is barely visible to the naked eye.

IV EQUIPMENT AND SUPPLIES

Consolidated lists of equipment, supplies, and culture media required for all multiple dilution tube tests described in this outline are shown in Table 3. Quantitative information is not presented; this is variable according to the extent of the testing procedure, the number of dilutions used, and the number of replicate tubes per dilution.



1 a Flame-sterilize an inoculation needle and air-cool.

b Dip the tip of the inoculation needle into the bacterial culture being studied.

c Streak the inoculation needle tip lightly back and forth over half the agar surface, as in (9), avoiding scratching or breaking the agar surface.

d Flame-sterilize the inoculation needle and air-cool.

2 a Turn the Petri dish one-quarter-turn and streak the inoculation needle tip lightly back and forth over one-half the agar surface, working from area (1) into one-half the unstreaked area of the agar.

b Flame-sterilize the inoculation needle and air-cool.

3 a Turn the Petri dish one-quarter-turn and streak the inoculation needle tip lightly back and forth over one-half the agar surface, working from area (20 into area (3), the remaining unstreaked area.

b Flame-sterilize the inoculation needle and set it aside.

c Close the culture container and incubate as prescribed.

Figure 3. A SUGGESTED PROCEDURE FOR COLONY ISOLATION BY A STREAK-PLATE TECHNIQUE

6-9

TABLE 3. APPARATUS AND SUPPLIES FOR STANDARD AND NONSTANDARD FERMENTATION TUBE TESTS

Description of Item	Total Coliform Group			Fecal Coliforms	
	Presumptive Test	Confirmed Test	Completed Test	(BALB)	(EC broth)
Lauryl tryptose broth or Lactose broth. 20 ml amounts of 1.5 × concentration medium, in 25 × 150 mm culture tubes with inverted fermentation vials, metal caps.	X				
Lauryl tryptose broth or Lactose broth. 10 ml amounts of single strength medium in 20 × 150 mm culture tubes with inverted fermentation vials, metal caps.	X		X		
Brilliant green lactose bile broth, 2% in 10 ml amounts, single strength, in 20 × 150 mm culture tubes with inverted fermentation vials, metal caps.		X			
Eosin methylene blue agar, poured in 100 × 15 mm Petri dishes		X	X		
Nutrient agar slants, in screw-capped tubes			X		
Boric acid lactose broth, 10 ml amounts of single strength medium in fermentation tubes.				X	
EC Broth, 10 ml amounts of single strength medium in fermentation tubes, metal caps.					X
Formate ricinoleate broth (provisional)			X		
Culture tube racks, 10 × 5 openings; each opening to accept 25 mm diameter tubes.	X	X	X	X	X
Pipettes, 10 ml, Mohr type, sterile, in metal cans.	X				
Pipettes, 2 ml (optional), Morh type, sterile, in metal cans	X				
Pipettes, 1 ml, Mohr type, sterile in metal cans	X				
Standard buffered dilution water, sterile, 99-ml amounts in screw-capped bottles.	X				
Gas burner, Bunsen type		X	X	X	X
Inoculation loop, loop 3-4 mm diameter, of nichrome or platinum-iridium wire, 26 B & S gauge, in suitable holder. (or sterile applicator stick)		X	X	X	X
Inoculation needle, nichrome, or platinum-iridium wire, 26 B & S gauge, in suitable holder.			X		
Incubator, adjusted to 35 ± 0.5°C	X	X	X		
Waterbath incubator, adjusted to 43 ± 0.5°C				X	
Waterbath incubator, adjusted to 44.0 - 45.0°C					X
Glass microscopic slides, 1" × 3"			X		
Slide racks (optional)			X		
Gram-stain solutions, complete set			X		
Compound microscope, oil immersion lens, Abbe' condenser			X		
Basket for discarded cultures	X	X	X	X	X
Container for discarded pipettes	X				

6-11

Part 2

DETAILED TESTING PROCEDURES FOR MEMBERS OF THE COLIFORM GROUP BY MULTIPLE DILUTION TUBE METHODS

I SCOPE

A Tests Described

1 Presumptive Test

2 Confirmed Test

3 Completed Test

4 Fecal Coliform Test

B Form of Presentation

The Presumptive, Confirmed, and Completed Tests are presented as total, independent procedures. It is recognized that this form of presentation is somewhat repetitious, inasmuch as the Presumptive Test is but preliminary to the Confirmed Test, and both the Presumptive Test and the Confirmed Test are preliminary to the Completed Test for total coliforms.

In using these procedures, the worker must know at the outset what is to be the stage at which the test is to be ended, and the details of the procedures throughout, in order to prevent the possibility of discarding gas-positive tubes before proper transfer procedures have been followed.

Thus, if the worker knows that the test will be ended at the Confirmed Test, he will turn at once to Section III, TESTING TO THE CONFIRMED TEST STAGE, and will ignore Sections II and IV.

The Fecal Coliform Test is described separately, in Section V, as an optional adjunct to the Confirmed Test and to the Completed Test.

II TESTING TO PRESUMPTIVE TEST STAGE

A First-Day Procedures

1 Prepare a laboratory data sheet for the sample. Record the following information: assigned laboratory number, source of sample, date and time of collection, temperature of the source, name of sample collector, date and time of receipt of sample in the laboratory. Also show the date and time of starting tests in the laboratory, name(s) of worker(s) performing the laboratory tests, and the sample volumes planted.

2 Label the tubes of lauryl tryptose broth required for the initial planting of the sample. The label should bear three identifying marks. The upper number is the identification of the worker(s) performing the test (applicable to personnel in SEC training courses), the number immediately below is the assigned laboratory number, corresponding with the laboratory record sheet. The lower number is the code to designate the sample volume and which tube of a replicate series is indicated.

NOTE: If 10-ml samples are being planted, be sure to use tubes containing the correct concentrations of culture medium. See Table 4 to adjust for the dilution factor.

3 Place the labeled culture tubes in an orderly arrangement in a culture tube rack, with the tubes intended for the largest sample volumes in the front row, and those intended for smaller volumes in the succeeding rows.

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TABLE 4

VOLUME-CONCENTRATION RELATIONSHIPS OF PRIMARY INOCULUM MEDIUM FOR INITIAL SAMPLE VOLUMES

Inoculum ml.	Amt. of Medium in Tube ml.	Volume of Medium and Inoculum ml.	Dehydrated Lactose Broth Required Grams/Liter
1 ml or less	10 ml.	10 ml.	13 g.
10	10	20	26.0 g.
10	20	30	19.5 g.

Inoculum ml.	Amt. of Medium in Tube ml.	Volume of Medium and Inoculum ml.	Dehydrated Lauryl Tryptose Lactose Broth Required Grams/Liter
1 ml or less	10 ml.	10 ml.	35.6 g.
10	10	20	71.2 g.
10	20	30	53.4 g.
10	30	40	47.3 g.

4 Shake the sample vigorously, approximately 25 times, in an up-and-down motion.

5 Measure the predetermined sample volumes into the labeled tubes of lauryl tryptose broth, using care to avoid introduction of any bacteria into the culture medium except those in the sample.

  a Use a 10-ml pipet for 10 ml sample portions, and 1-ml pipets for portions of 1 ml or less. Handle sterile pipets only near the mouthpiece, and protect the delivery end from external contamination.

  b When using the pipet to withdraw sample portions, do not dip the pipet more than 1/2 inch into the sample; otherwise sample running down the outside of the pipet will make measurements inaccurate.

6 After measuring all portions of the sample into their respective tubes of medium, gently shake the rack of inoculated tubes to insure good mixing of sample with the culture medium. Avoid vigorous shaking, as air bubbles may be shaken into the fermentation vials and thereby invalidate the test.

7 Place the rack of inoculated tubes in the incubator at 35° ± 0.5°C for 24 ± 2 hours.

B 24-hour Procedures

1 Remove the rack of lauryl tryptose broth cultures from the incubator, and shake gently. If gas is about to appear in the fermentation vials, the shaking will speed the process.

2 Examine each tube carefully. Record, in the column "24" under LST on the laboratory data sheet, each tube showing gas in the fermentation vial as a positive (+) test and each tube not showing gas as a negative (-) test. GAS IN ANY QUANTITY IS A POSITIVE TEST.

3 Discard all gas-positive tubes of lauryl tryptose broth, and return all the gas-negative tubes to the 35°C incubator for an additional 24 ± 2 hours.

C 48-hour Procedures

1 Remove the rack of culture tubes from the incubator, read and record gas production for each tube.

2 Be sure to record all results under the 48-hour LTB column on the data sheet. Discard all tubes. The Presumptive Test is concluded at this point, and Presumptive coliforms per 100 ml can be computed according to the methods described in Outline 21 of this Manual.

III TESTING TO CONFIRMED TEST STAGE

Note that the description starts with the sample inoculation and includes the Presumptive Test stage. The Confirmed Test preferred in FWPCA Laboratories is accomplished by means of the brilliant green lactose bile broth (BGLB) and the acceptable alternate tests are mentioned in III F. In addition, the Fecal Coliform Test is included as an optional adjunct to the procedure.

A First-Day Procedures

1 Prepare a laboratory data sheet for the sample. Record the following information: assigned laboratory number, source of sample, date and time of collection, temperature of the source, name of sample collector, date and time of receipt of sample in the laboratory. Also show the date and time of starting tests in the laboratory, name(s) of worker(s) performing the laboratory tests, and the sample volumes planted.

2 Label the tubes of lauryl tryptose broth required for the initial planting of the sample. The label should bear three identifying marks. The upper number is the identification of the worker(s) performing the test (applicable to personnel in training courses), the number immediately below is the assigned laboratory number, corresponding with the laboratory record sheet. The lower number is the code to designate the sample volume and which tube of a replicate series is indicated.

NOTE: If 10-ml samples are being planted, it is necessary to use tubes containing the correct concentration of culture medium. This has previously been noted in II A 2.

3 Place the labeled culture tubes in an orderly arrangement in a culture tube rack, with the tubes intended for the largest sample volumes in the front row, and those intended for smaller volumes in the succeeding rows.

4 Shake the sample vigorously, approximately 25 times, in an up-and-down motion.

5 Measure the predetermined sample volumes into the labeled tubes of lauryl tryptose broth, using care to avoid introduction of any bacteria into the culture medium except those in the sample.

   a Use a 10-ml pipet for 10 ml sample portions, and 1-ml pipets for portions of 1 ml or less. Handle sterile pipets only near the mouthpiece, and protect the delivery end from external contamination.

   b When using the pipet to withdraw sample portions, do not dip the pipet more than 1/2 inch into the sample; otherwise sample running down the outside of the pipet will make measurements inaccurate.

   c When delivering the sample into the culture medium, deliver sample portions of 1 ml or less down into the culture tube near the surface of the medium. Do not deliver small sample volumes at the top of the tube and allow them to run down inside the tube; too much of the sample will fail to reach the culture medium.

   d Prepare preliminary dilutions of samples for portions of 0.01 ml or less before delivery into the culture medium. See Table 1 for preparation of dilutions. NOTE: Always deliver diluted sample portions into the culture medium as soon as possible after preparation. The interval between preparation of dilution and introduction of sample into the medium never should be as much as 30 minutes.

6 After measuring all portions of the sample into their respective tubes of medium, gently shake the rack of inoculated tubes to insure good mixing of sample with the culture medium. Avoid vigorous shaking, as air bubbles may be shaken into the fermentation vials and thereby invalidate the test.

7 Place the rack of inoculated tubes in the incubator at $35^{\circ} \pm 0.5^{\circ}C$ for 24 ± 2 hours.

B 24-hour Procedures

1 Remove the rack of lauryl tryptose broth cultures from the incubator, and shake gently. If gas is about to appear in the fermentation vials, the shaking will speed the process.

2 Examine each tube carefully. Record, in the column "24" under LST on the laboratory data sheet, each tube showing gas in the fermentation vial as a positive (+) test and each tube not showing gas as a negative (-) test. GAS IN ANY QUANTITY IS A POSITIVE TEST.

3 Retain all gas-positive tubes of lauryl tryptose broth culture in their place in the rack, and proceed:

4 Select the gas-positive tubes of lauryl tryptose broth culture for Confirmed Test procedures. Confirmed Test procedures may not be required for all gas-positive cultures. If, after 24-hours of incubation, all five replicate cultures are gas-positive for two or more consecutive sample volumes, then select the set of five cultures representing the smallest volume of sample in which all tubes were gas-positive. Apply Confirmed Test procedures to all these cultures and to any other gas-positive cultures representing smaller volumes of sample, in which some tubes were gas-positive and some were gas-negative.

5 Label one tube of brilliant green lactose bile broth (BGLB) to correspond with each tube of lauryl tryptose broth selected for Confirmed Test procedures.

6 Gently shake the rack of Presumptive Test cultures. With a flame-sterilized inoculation loop transfer one loopful of culture from each gas-positive tube to the corresponding tube of BGLB. Place each newly inoculated culture into BGLB in the position of the original gas-positive tube.

7 After making the transfers, the rack should contain some 24-hour gas-negative tubes of lauryl tryptose broth and the newly inoculated BGLB.

8 If the Fecal Coliform Test is included in the testing procedures, consult Section V of this Part of the Outline of testing procedures.

9 Incubate the 24-hour gas-negative BGLB tubes and any newly-inoculated tubes of BGLB 24 ± 2 hours at 35° ± 0.5°C.

10 Discard all tubes of LTB and all 24-hour gas-positive BGLB cultures.

C 48-hour Procedures

1 Remove the rack of culture tubes from the incubator, read and record gas production for each tube.

2 Some tubes will be lauryl tryptose broth and some will be brilliant green lactose bile broth (BGLB). Be sure to record results from LTB under the 48-hour LTB column and the BGLB results under the 24-hour column of the data sheet.

3 Label tubes of BGLB to correspond with all (if any) 48-hour gas-positive cultures in lauryl tryptose broth. Transfer one loopful of culture from each gas-positive LTB culture to the correspondingly-labeled tube of BGLB. NOTE: All tubes of LTB culture which were negative at 24 hours and became positive at 48 hours are to be transferred. The option described above for 24-hour cultures does not apply at 48 hours.

4 If the Fecal Coliform Test is included in the testing procedure, consult Section V of the Part of the Outline of testing procedures.

5 Incubate the 24-hour gas-negative BGLB tubes and any newly-inoculated tubes of BGLB 24 ± 2 hours at 35° ± 0.5°C.

6 Discard all tubes of LTB and all 24-hour gas-positive BGLB cultures.

D 72-hour Procedures

1 If any cultures remain to be examined, all will be BGLB. Some may be 24 hours old and some may be 48 hours old. Remove such cultures from the incubator, examine each tube for gas production, and record results on the data sheet.

2 Be sure to record the results of 24-hour BGLB cultures in the "24" column under BGLB and the 48-hour results under the "48" column of the data sheet.

3 Return any 24-hour gas-negative cultures for incubation 24 ± 2 hours at 35 ± 0.5°C.

4 Discard all gas-positive BGLB cultures and all 48-hour gas-negative cultures from BGLB.

5 It is possible that all cultural work and results for the Confirmed Test have been finished at this point. If so, consult Outline 21 for codifying results and determination of Confirmed Test coliforms per 100 ml.

E 96-hour Procedures

At most only a few 48-hour cultures in BGLB may be present. Read and record gas production of such cultures in the "48" column under BGLB on the data sheet. Codify results and determine Confirmed Test coliforms per 100 ml as described in Outline 21.

F Streak-plate methods for the Confirmed Test, using eosin methylene blue agar or Endo agar plates, are accepted procedures in Standard methods. The worker who prefers to use one of these media in preference to BGLB (also approved in Standard Methods) is advised to refer to the current edition of "Standard Methods for the Examination of Water and Wastewater."

IV TESTING TO COMPLETED TEST STAGE

(Note that this description starts with the sample inoculation and proceeds through the Presumptive and the Confirmed Test stages. In addition, the Fecal Coliform Test is referred to as an optional adjunct to the procedure.)

A First-Day Procedures

1 Prepare a laboratory data sheet for the sample. Record the following information: assigned laboratory number, source of sample, date and time of collection, temperature of the source, name of sample collector, date and time of receipt of sample in the laboratory. Also show the date and time of starting tests in the laboratory, name(s) of worker(s) performing the laboratory tests, and the sample volumes planted.

2 Label the tubes of lauryl tryptose broth required for the initial planting of the sample. The label should bear three identifying marks. The upper number is the identification of the worker(s) performing the test (applicable to personnel in SEC training courses), the number immediately below is the assigned laboratory number, corresponding with the laboratory record sheet. The lower number is the code to designate the sample volume and which tube of a replicate series is indicated. Consult Table 2, this chapter, for guidance on labeling for laboratory data number and identification of individual tubes.

NOTE: If 10-ml samples are being plated, it is necessary to use tubes containing the correct concentration of culture medium. This has previously been noted in II A 2.

3 Place the labeled culture tubes in an orderly arrangement in a culture tube rack, with the tubes intended for the largest sample volumes in the front row, and those intended for smaller volumes in the succeeding rows.

4 Shake the sample vigorously, approximately 25 times, in an up-and-down motion.

5 Measure the predetermined sample volumes into the labeled tubes of lauryl tryptose broth, using care to avoid introduction of any bacteria into the culture medium except those in the sample.

a Use a 10-ml pipet for 10 ml sample portions, and 1-ml pipets for portions of 1 ml or less. Handle sterile pipets only near the mouthpiece, and protect the delivery end from external contamination.

b When using the pipet to withdraw sample portions, do not dip the pipet more than 1/2 inch into the sample; otherwise sample running down the outside of the pipet will make measurements inaccurate.

c When delivering the sample into the culture medium, deliver sample portions of 1 ml or less down into the culture tube near the surface of the medium. Do not deliver small sample volumes at the top of the tube and allow them to run down inside the tube; too much of the sample will fail to reach the culture medium.

d Prepare preliminary dilutions of samples for portions of 0.01 ml or less before delivery into the culture medium. See Table 1 for preparation of dilutions. NOTE: Always deliver diluted sample portions into the culture medium as soon as possible after preparation. The interval between preparation of dilution and introduction of sample into the medium never should be as much as 30 minutes.

6 After measuring all portions of the sample into their respective tubes of medium, gently shake the rack of inoculated tubes to insure good mixing of sample with the culture medium. Avoid vigorous shaking, as air bubbles may be shaken into the fermentation vials and thereby invalidate the test.

7 Place the rack of inoculated tubes in the incubator at 35° ± 0.5°C for 24 ± 2 hours.

B 24-hour Procedures

1 Remove the rack of lauryl tryptose broth cultures from the incubator, and shake gently. If gas is about to appear in the fermentation vials, the shaking will speed the process.

2 Examine each tube carefully. Record, in the column "24" under LST on the laboratory data sheet, each tube showing gas in the fermentation vial as a positive (+) test and each tube not showing gas as a negative (-) test. GAS IN ANY QUANTITY IS A POSITIVE TEST.

3 Retain all gas-positive tubes of lauryl tryptose broth culture in their place in the rack, and proceed.

4 Select the gas-positive tubes of lauryl tryptose broth culture for the Confirmed Test procedures. Confirmed Test procedures may not be required for all gas-positive cultures. If, after 24-hours of incubation, all five replicate cultures are gas-positive for two or more consecutive sample volumes, then select the set of five cultures representing the smallest volume of sample in which all tubes were gas-positive. Apply Confirmed Test procedures to all these cultures and to any other gas-positive cultures representing smaller volumes of sample, in which some tubes were gas-positive and some were gas-negative.

5 Label one tube of brilliant green lactose bile broth (BGLB) to correspond with each tube of lauryl tryptose broth selected for Confirmed Test procedures.

6 Gently shake the rack of Presumptive Test cultures. With a flame-sterilized inoculation loop transfer one loopful of culture from each gas-positive tube to the corresponding tube of BGLB. Place each newly inoculated culture into BGLB in the position of the original gas-positive tube.

7 If the Fecal Coliform Test is included in the testing procedure, consult Section V of this outline for details of the testing procedure.

8 After making the transfers, the rack should contain some 24-hour gas-negative tubes of lauryl tryptose broth and the newly inoculated BGLB. Incubate the rack of cultures at 35°C ± 0.5°C for 24 ± 2 hours.

C 48-hour Procedures

1 Remove the rack of culture tubes from the incubator, read and record gas production for each tube.

2 Some tubes will be lauryl tryptose broth and some will be brilliant green lactose bile broth (BGLB). Be sure to record results from LTB under the 48-hour LTB column and the BGLB results under the 24-hour column of the data sheet.

3 Label tubes of BGLB to correspond with all (if any) 48-hour gas-positive cultures in lauryl tryptose broth. Transfer one loopful of culture from each gas-positive LTB culture to the correspondingly-labeled tube of BGLB. NOTE: All tubes of LTB culture which were negative at 24 hours and became positive at 48 hours are to be transferred. The Option described above for 24-hour LTB cultures does not apply at 48 hours.

4 Incubate the 24-hour gas-negative BGLB tubes and any newly-inoculated tubes of BGLB 24 ± 2 hours at 35° ± 0.5°C. Retain all 24-hour gas-positive cultures in BGLB for further test procedures.

5 Label a Petri dish preparation of eosin methylene blue agar (EMB agar) to correspond with each gas-positive culture in BGLB.

6 Prepare a streak plate for colony isolation from each gas-positive culture in BGLB on the correspondingly-labeled EMB agar plate.

Incubate the EMB agar plates 24 ± 2 hours at 35° ± 0.5°C.

D 72-hour Procedures

1 Remove the cultures from the incubator. Some may be on BGLB; several EMB agar plates also can be expected.

2 Examine and record gas production results for any cultures in BGLB.

3 Retain any gas-positive BGLB cultures and prepare streak plate inoculations for colony isolation in EMB agar. Incubate the EMB agar plates 24 ± 2 hours at 35 ± 0.5°C. Discard the gas-positive BGLB cultures after transfer.

4 Reincubate any gas-negative BGLB cultures 24 ± 2 hours at 35° ± 0.5°C.

5 Discard all 48-hour gas-negative BGLB cultures.

6 Examine the EMB agar plates for the type of colonies developed thereon. Well-isolated colonies having a dark center (when viewed from the lower side, held toward a light) are termed "nucleated or fisheye" colonies, and are regarded as "typical" coliform colonies. A surface sheen may or may not be present on "typical" colonies. Colonies which are pink or opaque but are not nucleated are regarded as "atypical colonies." Other colony types are considered "noncoliform." Read and record results as + for "typical" (nucleated) colonies ± for "atypical" (non-nucleated pink or opaque colonies), and - for other types of colonies which might develop.

7 With plates bearing "typical" colonies, select at least one well-isolated colony and transfer it to a correspondingly-labeled tube of lactose broth and to an agar slant. As a second choice, select at least two "atypical" colonies (if typical colonies are not present) and transfer them to labeled tubes of lactose broth and to agar slants. As a third choice, in the absence of typical or atypical coliform-like colonies, select at least two well-isolated colonies representative of those appearing on the EMB plate, and transfer them to lactose broth and to agar slants.

8 Incubate all cultures transfered from EMB agar plates 24 ± 2 hours at 35 ± 0.5°C.

E 96-hour Procedures

1 Subcultures from the samples being studied may include: 48-hour tubes of BGLB, EMB agar plates, lactose broth tubes, and agar slant cultures.

2 If any 48-hour tubes of BGLB are present, read and record gas production in the "48" column under BGLB. From any gas-positive BGLB cultures prepare streak plate inoculations for colony isolation on EMB agar. Discard all tubes of BGLB, and incubate EMB agar plates 24 ± 2 hours at 35 ± 0.5°C.

3 If any EMB plates are present, examine and record results in the "EMB" column of the data sheet. Make transfers to agar slants and to lactose broth from all EMB agar plate cultures. In decreasing order of preference, transfer at least one typical colony, or at least two atypical colonies, or at least two colonies representative of those on the plate.

4 Examine and record results from the lactose broth cultures.

5 Prepare a Gram-stained smear from each of the agar slant cultures, as follows:

NOTE: Always prepare Gram stain from an actively growing culture, preferably about 18 hours old, and never more than 24 hours old. Failure to observe this precaution often results in irregular staining reactions.

a Thoroughly clean a glass slide to free it of any trace of oily film.

b Place one drop of distilled water on the slide.

c Use the inoculation needle to suspend a tiny amount of growth from the nutrient agar slant culture in the drop of water.

d Mix the thin suspension of cells with the tip of the inoculation needle, and allow the water to evaporate.

e "Fix" the smear by gently warming the slide over a flame.

f Stain the smear by flooding it for 1 minute with crystal violet solution.

g Flush the excess crystal violet solution off in gently running water, and gently blot dry with filter paper or with other clean absorbent paper.

h Flood the smear with Lugol's iodine for 1 minute.

i Wash the slide in gently running water and blot dry with filter paper.

j Decolorize the smear with 95% alcohol solution with gentle agitation for 10-30 seconds, depending upon extent of removal of crystal violet dye, then blot dry.

k Counterstain for 10 seconds with safranin solution, then wash in running water and blot dry.

l Examine the slide under the microscope, using the oil immersion lens. Coliform bacteria are Gram-negative, nonspore-forming, rod-shaped cells, occurring singly, in pairs, or rarely in short chains.

m If typical coliform staining reaction and morphology are observed, record + in the appropriate space under the "Gram Stain" column of the data sheet. If typical morphology and staining reaction are not observed, then mark it ± or -, and make suitable comment in the "remarks" column at the right-hand side of the data sheet.

n If spore-forming bacteria are observed, it will be necessary to repurify the culture from which the observations were made. Consult the instructor, or refer to Standard Methods, for procedures.

At this point, it is possible that all cultural work for the Completed Test has been finished. If so, codify results and determine Completed Test coliforms per 100 ml according to methods described in Outline 21.

F 120-hour Procedures and following:

1 Any procedures to be undertaken from this point are "straggler" cultures on media already described, and requiring step-by-step methodology already given in detail. Such cultures may be on: EMB plates, agar slants, or lactose broth. The same time-and-temperature of incubation required for earlier studies applies to the "stragglers" as do the observations, staining reactions, and interpretation of results. On conclusion of all cultural procedures, codify results and determine Completed Test coliforms per 100 ml as described in Outline 21.

V FECAL COLIFORM TEST

A General Information

1 The procedure described is an elevated temperature test for fecal coliform bacteria.

2 Equipment required for the tests are those required for the Presumptive Test of Standard Methods, a waterbath incubator, and the appropriate culture media.

B Fecal Coliform Test with EC Broth

1 Sample: The test is applied to gas-positive tubes from the Standard Methods Presumptive Test (lauryl tryptose broth), in parallel with Confirmed Test procedures.

2 24-hour Operations. Initial procedures are the planting procedures described for the Standard Methods Presumptive Coliform test.

a After reading and recording gas-production on lauryl tryptose broth, temporarily retain all gas-positive tubes.

b Label a tube of EC broth to correspond with each gas-positive tube of lauryl tryptose broth. The option regarding transfer of only a limited number of tubes to the Confirmed Test (see II, B, 5) sometimes can be applied here. However, the worker is urged to avoid exercise of this option until he has assured the applicability of the option by preliminary tests on the sample source.

c Transfer one drop of culture from each gas-positive culture in lauryl tryptose broth to the correspondingly labeled tube of EC broth.

d Incubate EC broth tubes 24 ± 2 hours at 44.0 - 45.0°C, in a waterbath with water depth sufficient to come up at least as high as the top of the culture medium in the tubes. Place in waterbath as soon as possible after inoculation and always within 30 minutes after inoculation.

3 48-hour operations

a Remove the rack of LTB cultures from the waterbath, shake gently, and record gas production for each tube. Gas in any quantity is a positive test.

b As soon as results are recorded, discard all tubes. (This is a 24-hour test for EC broth inoculations and not a 48-hour test.)

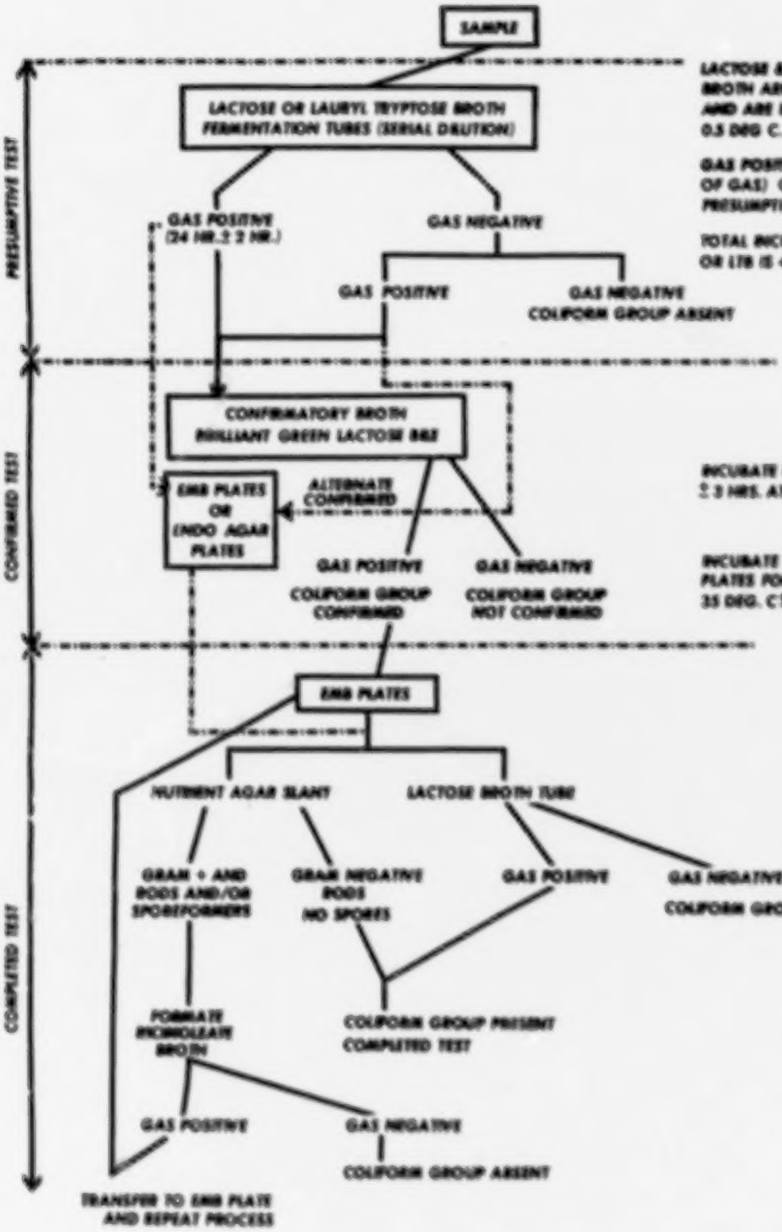
c Transfer any additional 48-hour gas-positive tubes of lauryl tryptose broth to correspondingly labeled tubes of EC broth. Incubate 24 ± 2 hours at 44.0 - 45.0°C.

4 72-hour operations

a Read and record gas production for each tube. Discard all cultures.

b Based on the principles described in Chapter 20, codify results and determine fecal coliform count per 100 ml of sample.

TESTS FOR COLIFORM GROUP


SAMPLE
PRESUMPTIVE TEST
LACTOSE OR LAURYL TRYPTOSE BROTH FERMENTATION TUBES (SERIAL DILUTION)
GAS POSITIVE (24 HR.± 2 HR.)
GAS NEGATIVE
GAS POSITIVE
GAS NEGATIVE COLIFORM GROUP ABSENT
CONFIRMED TEST
CONFIRMATORY BROTH BRILLIANT GREEN LACTOSE BILE
EMB PLATES OR ENDO AGAR PLATES
ALTERNATE CONFIRMED
GAS POSITIVE
COLIFORM GROUP CONFIRMED
GAS NEGATIVE
COLIFORM GROUP NOT CONFIRMED
COMPLETED TEST
EMB PLATES
NUTRIENT AGAR SLANT
LACTOSE BROTH TUBE
GRAM + AND RODS AND/OR SPOREFORMERS
GRAM NEGATIVE RODS NO SPORES
GAS POSITIVE
GAS NEGATIVE
COLIFORM GROUP ABSENT
FORMATE RICINOLEATE BROTH
COLIFORM GROUP PRESENT COMPLETED TEST
GAS POSITIVE
GAS NEGATIVE
COLIFORM GROUP ABSENT
TRANSFER TO EMB PLATE AND REPEAT PROCESS

LACTOSE & LAURYL TRYPTOSE BROTH ARE INTERCHANGEABLE MEDIA AND ARE INCUBATED AT 35 DEG C± 0.5 DEG C.

GAS POSITIVES TUBES (ANY AMOUNT OF GAS) CONSTITUTE A POSITIVE PRESUMPTIVE TEST

TOTAL INCUBATION TIME FOR LACTOSE OR LTB IS 48 HRS.± 3 HRS.

INCUBATE BGLB TUBES FOR 48 HRS. ± 3 HRS. AT 35 DEG. C± 0.5 DEG. C.

INCUBATE EMB OR ENDO AGAR PLATES FOR 24 HRS.± 2 HRS AT 35 DEG. C± 0.5 DEG. C.

Part 3
LABORATORY METHODS FOR FECAL STREPTOCOCCUS
(Day-By-Day Procedures)

I GENERAL INFORMATION

A The same sampling and holding procedures apply as for the coliform test.

B The number of fecal streptococci in water generally is lower than the number of coliform bacteria. It is good practice in multiple dilution tube tests to start the sample planting series with one sample increment larger than for the coliform test. For example: If a sample planting series of 1.0, 0.1, 0.01, and 0.001 ml is planned for the coliform test, it is suggested that a series of 10, 1.0, 0.1, and 0.01 ml be planted for the fecal streptococcus test.

C Equipment required for the test is the same as required for the Standard Methods Presumptive and Confirmed Tests, except for the differences in culture media.

II STANDARD METHODS (Tentative) PROCEDURES

A First-Day Operations

1 Prepare the sample data sheet and labeled tubes of azide dextrose broth in the same manner as for the Presumptive Test. NOTE: If 10-ml samples are included in the series, be sure to use a special concentration (ordinarily double-strength) of azide dextrose broth for these sample portions.

2 Shake the sample vigorously, approximately 25 times, in an up-and-down motion.

3 Measure the predetermined sample volumes into the labeled tubes of azide dextrose broth, using the sample measurement and delivery techniques used for the Presumptive Test.

4 Shake the rack of tubes of inoculated culture media, to insure good mixing of sample with medium.

5 Place the rack of inoculated tubes in the incubator at 35° ± 0.5°C for 24 ± 2 hours.

B 24-hour Operations

1 Remove the rack of tubes from the incubator. Read and record the results from each tube. Growth is a positive test with this test. Evidence of growth consists either of turbidity of the medium, a "button" of sediment at the bottom of the culture tube, or both.

2 Label a tube of ethyl violet azide broth to correspond with each positive culture of azide dextrose broth. It may be permissible to use the same confirmatory option as described for the coliform Confirmed Test, in this Outline.

3 Shake the rack of cultures gently, to resuspend any living cells which have settled out to the bottom of the culture tubes.

4 Transfer three loopfuls of culture from each growth-positive tube of azide dextrose broth to the correspondingly labeled tube of ethyl violet azide broth.

5 As transfers are made, place the newly inoculated tubes of ethyl violet azide broth in the positions in the rack formerly occupied by the growth-positive tubes of azide dextrose broth. Discard the tubes of azide dextrose broth culture.

6 Return the rack, containing 24-hour growth-negative azide dextrose broth tubes and newly-inoculated tubes of ethyl violet azide broth, to the incubator. Incubate 24 ± 2 hours at 35° ± 0.5°C.

C 48-hour Operations

1 Remove the rack of tubes from the incubator. Read and report results. Growth, either in azide dextrose broth or in ethyl violet azide broth, is a positive test. Be sure to report the results of the azide dextrose broth medium under the "48" column for that medium and the results of the ethyl violet azide broth cultures under the "24" column for that medium.

2 Any 48-hour growth-positive cultures of azide dextrose broth are to be transferred (three loopsful) to ethyl violet azide broth. Discard all 48-hour growth-negative tubes of azide dextrose broth and all 24-hour growth-positive tubes of ethyl violet azide broth.

3 Incubate the 24-hour growth-negative and the newly-inoculated tubes of ethyl violet azide broth 24 ± 2 hours at 35° ± 0.5°C.

D 72-hour Operations

1 Read and report growth results of all tubes of ethyl violet azide broth.

2 Discard all growth-positive cultures and all 48-hour growth-negative cultures.

3 Reincubate any 24-hour growth-negative cultures in ethyl violet azide broth 24 ± 2 hours at 35° ± 0.5°C.

E 96-hour Operations

1 Read and report growth results of any remaining tubes of ethyl violet azide broth.

2 Codify results and determine fecal streptococci per 100 ml according to the procedures described in Outline 21.

REFERENCES

1 Standard Methods for the Examination of Water and Wastewater (12th ed). Prepared and Published Jointly by American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 1965.

2 Geldreich, E.E., Clark, H.F., Kabler, P.W., Huff, C.B., and Bordner, R.H. The Coliform Group. II. Reactions in EC Medium at 45°C. Appl. Microbiol. 8:347-348. 1958.

3 Geldreich, E.E., Bordner, R.H., Huff, C.B., Clark, H.F., and Kabler, P.W. Type Distribution of Coliform Bacteria in the Feces of Warm-Blooded Animals. J. Water Pollution Control Federation. 34:295-301. 1962.

4 Recommend Proc. for the Bacteriological Examination of Sea Water and Shellfish. 3rd Edition, American Public Health Association. 1962.

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MEMBRANE FILTER LABORATORY AND FIELD PROCEDURES

I BASIC PROCEDURES

A Introduction

Successful application of membrane filter methods requires development of good routine operational practices. The detailed basic procedures described in this Section are applicable to all membrane filter methods in water bacteriology for filtration, incubation, colony counting, and reporting of results. In addition, equipment and supplies used in all membrane filter procedures are described here and not repeated elsewhere in such detail.

Workers using membrane filter methods for the first time are urged to become thoroughly familiar with these basic procedures and precautions.

B General Supplies and Equipment List

Table 1 is a check list of materials.

C "Sterilizing" Media

Set tubes in a boiling waterbath for 10 minutes. This method suffices for medium in tubes up to 25 × 150 mm.

Alternately, heat coliform media to the first bubble of boiling. Stir the medium frequently if direct heat is used, to avoid charring the medium.

Do not autoclave.

D General Laboratory Procedures with Membrane Filters

1 Prepare data sheet

Show sample identification, test performed including media and methods, sample filtration volumes, and the bench numbers assigned to individual membrane filters.

2 Disinfect the laboratory bench surface.

Use a suitable disinfectant solution and allow the surface to dry before proceeding.

3 Set out culture containers in an orderly arrangement.

4 Label the culture containers.

Numbers correspond with the filter numbers shown on the data sheet.

5 Place one sterile absorbent pad in each culture container, unless an agar medium is being used.

Use sterile forceps for all manipulations of absorbent pads and membrane filters. Forceps sterility is maintained by storing the working tips in about 1 inch of methanol or ethanol. Because the alcohol deteriorates the filter, dissipate it by burning before using the forceps. Avoid heating the forceps in the burner as hot metal chars the filter.

6 Deliver enough culture medium to saturate each absorbent pad, using a sterile pipette.

Exact quantities cannot be stated because pads vary. Sufficient medium should be applied so that when the culture container is tipped, a good-sized drop of culture medium freely drains out of the absorbent pad.

When an agar medium is used, absorbent pads are not used. The amount of medium should be sufficient to make a layer approximately 1/8" deep in the culture container. In the 50 mm

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the FWPCA and the U.S. Department of the Interior.

7-2

Table 1. EQUIPMENT, SUPPLIES AND MEDIA

Item	Standard Tests		Nonstandard Tests			
	M-Endo Broth	L. E. S. Coliform	Delayed Coliform	Fecal Coliform	Fecal Streptococcus	Verified Test
Funnel unit assemblies	X	X	X	X	X	
Ring stand, with about a 3" split ring, to support the filtration funnel.	X	X	X	X	X	
Forceps, curved-end round tipped, special type for MF work.	X	X	X	X	X	
Methanol, in small wide-mouthed bottles, about 20 ml for sterilizing forceps.	X	X	X	X	X	
Suction flasks, glass, 1 liter, mouth to fit No. 8 stopper.	X	X	X	X	X	
Rubber tubing, 2-3 feet, to connect suction flask to vacuum services, latex rubber 3/16" I. D. by 3/32" wall.	X	X	X	X	X	
Pinch clamps strong enough for tight compression of rubber tubing above.	X	X	X	X	X	
Pipettes, 10 ml, graduated, Mohr type, sterile, dispense 10 per can per working space per day. (Resterilize daily to meet need).	X	X	X	X	X	
Pipettes, 1 ml, graduated, Mohr type, sterile, dispense 24 per can per working space per day. (Resterilize daily to meet need).	X	X	X	X	X	
Pipette boxes, sterile, for 1 ml and 10 ml pipettes (sterilize above pipettes in these boxes).	X	X	X	X	X	
Cylinders, 100 ml graduated, sterile, (resterilize daily to meet need).	X	X	X	X	X	
Jars, to receive used pipettes.	X	X	X	X	X	
Gas burner, Bunsen or similar laboratory type.	X	X	X	X	X	
Wax pencils, red, suitable for writing on glass	X	X	X	X	X	
Sponge in dilute iodine, to wipe down the desk tops.	X	X	X	X	X	
Membrane filters (white, grid marked, sterile, and suitable pore size for microbiological analysis of water)	X	X	X	X	X	
Absorbent pads for nutrient, (47 mm in diameter), sterile, in units of 10 pads per package.	X	X	X	X	X	
Petri dishes, disposable, plastic, 50 × 12 mm, sterile.	X	X	X	X	X	
Waterbath incubator 45.5 ± .5°C.				X		
Vegetable crispers, or cake boxes, plastic, with tight fitting covers, for membrane filter incubations.	X	X	X		X	
Fluorescent lamp, with extension cord equipped with a simple lens of about 4X magnification.	X	X	X	X	X	
Ring stand, with clamps, utility type	X	X	X	X	X	
Half-round glass paper weights for colony counting, with lower half of a 2-oz metal ointment box.	X	X	X	X	X	
Hand tally, single unit acceptable, hand or desk type.	X	X	X	X	X	
Stereoscopic (dissection) microscope, magnification of 10X or 15X, preferable binocular wide field type.	X	X	X	X	X	

7-3

Table 1. EQUIPMENT, SUPPLIES AND MEDIA (Cont'd)

Item	Standard Tests		Nonstandard Tests			
	M-Endo Broth	L. E. S. Coliform	Delayed Coliform	Fecal Coliform	Fecal Streptococcus	Verified Test
Bacteriological inoculating needle.						X
Wire racks for culture tubes, 10 openings by five openings preferred, dimensions overall approximately 6" × 12".						X
Phenol Red Lactose Broth in 20 × 150 mm fermentation tubes with metal caps, 10 ml per tube.						X
Eosin Methylene Blue Agar (Levine) in petri plates, prepared ready for use.						X
Nutrient agar slants, in screw capped tubes, 16 × 126 mm.						X
Gram stain solutions, 4 solutions per complete set.						X
Microscope, compound, binocular, with oil immersion lens, microscope lamp and immersion oil.						X
Microscope slides, new, clean, 1" × 3" size.						X
Water proof plastic bags, 3 × 7 1/ 4" for fecal coliform culture dish incubation.				X		
M-Endo medium, MF dehydrated medium in 25 × 95 mm flat bottomed screw-capped glass vials, 1.44 g per tube, sufficient for 30 ml of medium.	X		X			
Ethanol, 95% in small bottles or screw-capped tubes, about 20 ml per tube.	X	X	X			
Sodium benzoate solution, 12% aqueous, in 25 × 150 mm screw-capped tubes, about 10 ml per tube.		X				
L. E. S. Endo Agar MF, dehydrated M-Endo medium, 0.36 g per 25 × 95 mm flat bottomed screw-capped glass vial, plus 0.45 g agar, for 30 ml.		X				
Lactose Lauryl Sulfate Tryptose Broth in 25 × 150 mm test tube without included gas tube, about 25 ml, for enrichment in L. E. S. method.		X				
M-FC Broth for fecal coliform, dehydrated medium in 25 × 95 mm flat bottomed screw-capped glass vials, 1.23 g per tube, sufficient for 30 ml of culture medium.				X		
Rosolic acid, 1% solution, in 0.2N NaOH, in 25 × 150 mm flat bottomed screw-capped tubes, about 5 ml per tube, freshly prepared.				X		
M-Enterococcus Agar, dehydrated medium in 25 × 150 mm screw-capped tubes, sufficient for 30 ml, 1.26 g per tube.					X	
Dilution bottles, 6-oz, preferable boro-silicate glass, with screw-cap (or rubber stopper protected by paper), each containing 99 ml of phosphate buffered dilution water.	X	X	X	X	X	
Electric hot plate surface	X	X	X	X	X	
Beakers, 400 - 600 ml (for water-bath in preparation of membrane filter culture media).	X	X	X	X	X	
Crucible tongs, to be used at electric hot plates, for removal of hot tubes of culture media from boiling waterbath.	X	X	X	X	X	

plastic culture containers this corresponds to approximately 6-8 ml of culture medium.

7 Organize supplies and equipment for convenient sample filtration. In training courses, laboratory instructors will suggest useful arrangements; eventually the individual will select a system of bench-top organization most suited to his own needs. The important point in any arrangement is to have all needed equipment and supplies conveniently at hand, in such a pattern as to minimize lost time in useless motions.

8 Lay a sterile membrane filter on the filter holder, grid-side up, centered over the porous part of the filter support plate.

Membrane filters are extremely delicate and easily damaged. For manipulation, the sterile forceps should always grasp the outer part of the filter disk, outside the part of the filter through which the sample passes.

9 Attach the funnel element to the base of the filtration unit.

To avoid damage to the membrane filter, the funnel element never should be turned or twisted while being seated and locked to the lower element of the filter holding unit. Filter holding units featuring a bayonet joint and locking ring to join the upper element to the lower element require special care on the part of the operator. The locking ring should be turned sufficiently to give a snug fit, but should not be tightened excessively.

10 Shake the sample thoroughly.

11 Measure sample into the funnel with vacuum turned off.

The primary objectives here are: 1) accurate measurement of sample; and 2) optimum distribution of colonies on the filter after incubation. To meet these objectives, methods of measurement and dispensation to the filtration assembly are varied with different sample filtration volumes.

a With samples greater than 20 ml, measure the sample with a sterile graduated cylinder and pour it into the funnel.

b With samples of 10 ml to 20 ml, measure the sample with a sterile 10 ml or 20 ml pipette, and pipette on a dry membrane in the filtration assembly.

c With samples of 2 ml to 10 ml, pour about 20 ml of sterile dilution water into the filtration assembly, then measure the sample into the sterile buffered dilution water with a 10 ml sterile pipette.

d With samples of 0.5 to 2 ml, pour about 20 ml of sterile dilution water into the funnel assembly, then measure the sample into the sterile dilution water in the funnel with a 1 ml or a 2 ml pipette.

e If a sample of less than 0.5 ml is to be filtered, prepare appropriate dilutions in sterile dilution water, and proceed as applicable in item c or d above.

When dilutions of samples are needed, always make the filtrations as soon as possible after dilution of the sample; this never should exceed 30 minutes. Always shake sample dilutions thoroughly before delivering measured volumes.

12 Turn on the vacuum.

Open the appropriate spring clamp or valve, and filter the sample.

13 Rinse the sample through the filter.

After all the sample has passed through the membrane filter, rinse down the sides of the funnel walls with at least 20 ml of sterile dilution water. Repeat the rinse twice after all the first rinse has passed through the filter. Cut off suction on the filtration assembly.

After sample filtration a few droplets of sample usually remain adhered to the funnel walls. Unless these droplets are removed, the bacteria contained in them will be a source of contamination of water samples. (In laboratory practice the funnel unit is not routinely sterilized between successive filtrations of a series). The purpose of the funnel rinse is to flush all droplets of a sample from the funnel walls to the membrane filter. Extensive tests have shown that with proper rinsing technique, bacterial retention on the funnel walls is negligible.

14 Remove the funnel element of the filter holding unit.

If a ring stand and split ring is used, hang the funnel element on the ring; otherwise place the funnel unit, inverted, on the bench surface.

15 Take the membrane filter from the filter holder and carefully place it, grid-side up on the medium.

Check that no air bubbles have been trapped between the membrane filter and the underlying absorbent pad or agar. Relay the membrane if necessary.

16 Place in incubator after finishing filtration series.

Invert the containers. The immediate atmosphere of the incubating membrane filter must be at or very near 100% relative humidity.

17 Count colonies which have appeared after incubating for the prescribed time.

A stereoscopic microscope magnifying 10-15 times and careful illumination give best counts.

For reporting results, the computation is:

$$\text{bacteria/100 ml} = \frac{\text{No. colonies counted} \times 100}{\text{Sample volume filtered in ml}}$$

Example:

A total of 36 colonies grew after filtering a 10 ml sample. The number reported is:

$$\frac{36 \text{ colonies}}{10 \text{ ml}} \times 100 = 360 \text{ per } 100 \text{ ml}$$

II MF LABORATORY TESTS FOR COLIFORM GROUP

A Standard Coliform Test (Based on M-Endo Broth MF)

Standard counts require two additions to the general procedure. First is characteristic medium. Second is the critical lighting necessary to count the golden sheen colonies of coliform bacteria.

1 Culture medium

a M-Endo Broth MF Difco 0749-02 or the equivalent BBL M-Coliform Broth 01-494

Preparation of Culture Medium (M-Endo Broth) for Standard MF Coliform Test

Yeast extract	1.5	g
Casitone or equivalent	5.0	g
Thiopeptone or equivalent	5.0	g
Tryptose	10.0	g
Lactose	12.5	g
Sodium desoxycholate	0.1	g
Dipotassium phosphate	4.375	g
Monopotassium phosphate	1.375	g
Sodium chloride	5.0	g
Sodium lauryl sulfate	0.05	g
Basic fuchsin (bacteriological)	1.05	g
Sodium sulfite	2.1	g
Distilled water (containing 20.0 ml ethanol)	1000	ml

This medium is available in dehydrated form and it is recommended that the commercially available medium be used in preference to compounding the medium of its individual constituents.

To prepare the medium for use, suspend the dehydrated medium at the rate of 48 grams per liter of water containing ethyl alcohol at the rate of 20 ml per liter.

As a time-saving convenience, it is recommended that the laboratory worker preweigh the dehydrated medium in closed tubes for several days, or even weeks, at one operation.

With this system, a large number of increments of dehydrated medium (e.g., 1.44 grams), sufficient for some convenient (e.g., 30 ml) volume of finished culture medium are weighed and dispensed into screw-capped culture tubes, and stored until needed.

A supply of distilled water containing 20 ml stock ethanol per liter is maintained.

When the medium is to be used, it is reconstituted by adding 30 ml of the distilled water-ethanol mixture per tube of pre-weighed dehydrated culture medium.

b Medium is "sterilized" as directed in I, C.

c Finished medium can be retained up to 96 hours if kept in a cool, dark place. Many workers prefer to reconstitute fresh medium daily.

2 Filtration and incubation procedures are as given in I, D.

Special instructions:

a For counting, use the wide field binocular dissecting microscope, or simple lens. For illumination, use a light source perpendicular to the plane of the membrane filter. A small fluorescent lamp is ideal for the purpose.

b Coliform colonies are red or pink and have a "metallic" surface sheen under reflected light which may cover the entire colony, or it may appear only in the center. Non-coliform colonies range from colorless to pink, but do not have the characteristic sheen.

c Record the colony counts on the data sheet, and compute the coliform count per 100 ml of sample.

B Standard Coliform Tests (Based on L.E.S. Endo Agar)

The distinction of the L.E.S. count is a two hour enrichment incubation on LST broth. M-Endo medium is used as agar rather than the broth.

1 Preparation of culture medium (L.E.S. Endo Agar) for L.E.S. coliform test

a Formula from McCarthy, Delaney, and Grasso(2)

Bacto-Yeast Extract	1.2	g
Bacto-Casitone	3.7	g
Bacto-Thiopeptone	3.7	g
Bacto-Tryptose	7.5	g
Bacto-Lactose	9.4	g
Dipotassium phosphate	3.3	g
Monopotassium phosphate	1.0	g
Sodium chloride	3.7	g
Sodium desoxycholate	0.1	g
Sodium lauryl sulfate	0.05	g
Sodium sulfite	1.6	g
Bacto-Basic fuchsin	0.8	g
Agar	15	g
Distilled water (containing 20 ml ethyl alcohol)	1000	ml

b To rehydrate the medium, suspend 51 grams in the water-ethyl alcohol sodium.

c Medium is "sterilized" as directed in I, C.

d Pour 4-6 ml of melted L.E.S. Agar into the smaller half of the container. The liquid medium should be at 45-50°C. Allow the medium to cool and solidify.

e If the containers with medium are stored in the refrigerator in the dark, they are usable over a two week period.

2 Procedures for filtration and incubation

a Lay out the culture dishes in a row or series of rows as usual.

b Place one sterile absorbent pad in the larger half of each container. Use sterile forceps for all manipulations of the pads. (Agar occupies smaller half.)

c Using a sterile pipette, deliver enough single strength lauryl sulfate tryptose broth to saturate the pad only. Excess interferes.

d Follow general procedures for filtering in I, D. Place filters on pad with lauryl sulfate tryptose broth.

e Upon completion of the filtrations, invert the culture containers and incubate at 35°C for 2 ± 1/2 hours.

3 2-hour procedures

a Transfer the membrane filter from the enrichment pad in the upper half to the agar medium in the lower half of the container. Carefully roll the membrane onto the agar surface to avoid trapping air bubbles beneath the membrane.

b Removal of the used absorbent pad is optional.

c The container is inverted and incubated 22 hours at 35°C.

4 Counting procedures are as in I, D.

5 L.E.S. Endo Agar may be used as a single-stage medium (no enrichment step) in the same manner as M-Endo Broth, MF.

C Delayed Incubation Coliform Test

The delayed coliform count requires two media and permits 24-72 hour delay before incubating for counts.

1 Preparation of culture media for delayed incubation coliform test

a Preservative media
M-Endo Broth base

To a 30 ml tube of M-Endo Broth MF prepared in accordance with directions in II, A, 1 of this outline, add 1.0 ml of 12% aqueous solution of sodium benzoate after sterilizing.

LES MF Holding Medium - Coliform: Dissolve 12.7 grams in 1 liter of distilled water. No heating is necessary. Final pH 7.1 ± 0.1.

b Growth media

M-Endo Broth MF is used, prepared as described in II, A, 1 earlier in this outline. Alternately, L.E.S. Endo Medium may be used.

2 General filtration followed is in I, D.

Special procedures are:

a Transfer the membrane filter from the filtration apparatus to a pad saturated with benzoated M-Endo Broth.

b Close the culture dishes and store membrane filters at room temperature. If the cultures are transmitted to a central laboratory for conclusion of test, then check the identification markings on each culture container against the transmittal sheet. Each culture and the transmittal sheet should show at least:

The date the sample was collected and filtered

The source of sample

Serial number of filtration

Quantity of water filtered.

c Place the culture containers and transmittal sheet in a mailing carton and immediately send it to the central laboratory. Transportation time should not exceed 3 days, and preferably less.

d On receipt in the central laboratory, unpack mailing carton, and lay out the culture containers on the laboratory bench.

e Remove the tops from the culture containers. Using sterile forceps, remove each membrane and its absorbent pad to the other half of the culture container. (See j, below, for alternate procedure.)

f With a sterile pipette or sterile absorbent pad, remove preservative medium from the culture container.

g Place a sterile absorbent pad in each culture container, and deliver enough freshly prepared M-Endo Broth to saturate each pad.

h Using sterile forceps, transfer the membrane to the new absorbent pad containing M-Endo Broth. Place the membrane carefully to avoid entrapment of air between the membrane and the underlying absorbent pad. Discard the absorbent pad containing preservative medium.

i After incubation 24 hours at 35°C, count colonies as in the above section A, 2.

j If L.E.S. Endo Agar is used, the steps beginning with (e) above are omitted; and the membrane filter is removed from the preservative medium and transferred to a fresh culture container with L.E.S. Endo Agar, incubated, and colonies counted in the usual way.

D Verified Membrane Filter Coliform Test

This procedure applies to identification of colonies growing on Endo-type media used for determination of total coliform counts. Isolates from these colonies are studied for gas production from lactose and typical coliform morphology. In effect, the procedure corresponds with the Completed Test stage of the multiple fermentation tube test for coliforms.

Procedure:

1 Select a membrane filter bearing several well-isolated coliform-type colonies.

2 Using sterile technique, pick all colonies in a selected area with the inoculation needle, making transfers into tubes of phenol red lactose broth (or lauryl sulfate tryptose lactose broth). Use a data sheet similar to the sheet titled "Verified Membrane Filter Test" in the laboratory section of this manual. Record the interpretation of each colony, "C" for colonies having the typical color and sheen of coliforms; "NC" for colonies not conforming to coliform colony appearance on Endo-type media.

3 Incubate the broth tubes at 35°C.

4 At 24 hours:

  a Read and record the results from the lactose broth fermentation tubes. The following code is suggested:

Code	Result
O	No indication of acid or gas production, either with or without evidence of growth.
A	Evidence of acid but not gas (applies only when a pH indicator is included in the broth medium)
G	Growth with production of gas. If pH indicator is used, use symbol AG to show evidence of acid. Gas in any quantity is a positive test.

b Tubes not showing gas production are returned to the 35°C incubator.

c Gas-positive tubes are transferred as follows:

1) Prepare a streak inoculation on EMB agar for colony isolation.

2) Inoculate a single nutrient agar slant from each gas-positive tube.

3) Incubate at 35°C.

5 At 48 hours:

a Read and record results of lactose broth tubes which were negative at 24 hours and were returned for further incubation.

b Gas-positive cultures are subjected to further transfers as in 4c, above. Gas-negative cultures are discarded without further study; they are coliform-negative.

c Examine the cultures transferred to EMB agar plates and to nutrient agar slants, as follows:

1) Examine the EMB agar plate for evidence of purity of culture; if the culture represents more than one colony type, discard the nutrient agar culture and reisolate each of the representative colonial types on the EMB plate and resume as with 4c above for each isolation. If purity of culture appears evident, continue with c (2) below.

2) Prepare a smear and Gram stain from each nutrient agar slant culture. The Gram stain should be made on a culture not more than 24 hours old. Examine under oil immersion for typical coliform morphology, and record results.

6 At 72 hours:

Perform procedures described in 5c above, and record results.

7 Coliform colonies are considered verified if the procedures demonstrate a pure culture of bacteria which are gram negative nonspore-forming rods and produce gas from lactose at 35°C.

E Fecal Coliform Count (Based on M-FC Broth Base)

The count depends upon growth on a special medium at 44.5 ± .5°C.

1 Preparation of Culture Medium (M-FC Broth Base) for Fecal Coliform Count

a Composition

Tryptose	10.0 g
Proteose Peptone No. 3	5.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Lactose	12.5 g
Bile salts No. 3	1.5 g
Rosolic acid* (Allied Chemical)	10.0 ml
Aniline blue (Allied Chemical)	0.1 g
Distilled water	1000 ml

b To prepare medium dissolve 37.1 grams in a liter of distilled water which contains 10 ml of 1% rosolic acid in 0.2 N NaOH.

Fresh solutions of rosolic acid give best results. Discard solutions which have changed from dark red to orange.

c To sterilize, heat to boiling as directed in I, C.

d Prepared medium may be retained up to 4 days in the dark at 2-8°C.

2 Special supplies

Small water proof plastic sacks capable of being sealed against water with capacity of 3 to 6 culture containers.

3 Filtration procedures are as given in I, D.

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*Prepare 1% solution of rosolic acid in 0.2 N NaOH. This dye is practically insoluble in water.

4 Elevated temperature incubation

a Place fecal coliform count membranes at 44.5 ± 0.5°C as rapidly as possible.

Filter membranes for fecal coliform counts consecutively and immediately place them in their containers. Insert as many as six containers into the sacks and seal. Tear off the perforated top, grasp the side wires, and twirl the sack to roll the open end inside the folds of sack. Then submerge the sacks with containers beneath the surface of a 44.5 ± .5°C waterbath.

b Incubate for 22 ± 2 hours.

5 Counting procedures

Examine and count colonies as follows:

a Use a wide field binocular dissecting microscope with 5 - 10X magnification.

b Low angle lighting from the side is advantageous.

c Fecal coliform colonies are blue, generally 1-3 mm in diameter.

d Record the colony counts on the data sheet, and report the fecal coliform count per 100 ml of sample. (I, D, 17 illustrates method)

III TESTS FOR FECAL STREPTOCOCCAL GROUP-MEMBRANE FILTER METHOD

A 48 hour incubation period on a choice of two different media, giving high selectivity for fecal streptococci, are the distinctive features of the tests.

A Test for Members of Fecal Streptococcal (Tentative, Standard Methods) M-Enterococcus Agar Medium

1 Preparation of the culture medium

a Formula (The Difco formula is shown, but equivalent constitutents from other sources are equally acceptable).

Bacto tryptose	20.0 g
Bacto yeast extract	5.0 g
Bacto dextrose	2.0 g
Dipotassium phosphate	4.0 g
Sodium Azide	0.4 g
Bacto agar	10.0 g
2, 3, 5, Triphenyl tetrazolium chloride	0.1 g

b The medium is prepared by rehydration at the rate of 42 grams per 1000 ml of distilled water. It is recommended that the medium in dehydrated form be preweighed and dispensed into culture containers (about 25 × 150 mm) in quantities sufficient for preparation of 30 ml of culture medium (1.26 g per tube).

c Follow I, C, for "sterilizing" heat medium. The medium should be cooled 45-50°C before being dispensed into culture containers.

2 List of apparatus, materials, as given in Table I.

3 Procedure, in general, as given in I.

Special instructions

a To prepare agar plates, open the containers, and pour 5-8 ml of sterile melted M-Enterococcoccus agar into each. Replace the covers of the culture containers, and allow them to stand until the agar has solidified.

b Incubate for 48 hours, inverted, with 100% relative humidity, after filtrations are completed. If the entire incubator does not have saturated humidity, acceptable conditions can be secured by placing the cultures in a tightly closed container with wet paper, towels, or other moist material.

c After incubation, remove the cultures from the incubator, and count all colonies under wide field binocular dissecting microscope with magnification set at 10X or 20X. Fecal streptococcus colonies are 0.5 - 2 mm in diameter, and flat to raised smooth, and vary from pale pink to dark red in color.

d Report enterococcus count per 100 ml of sample. This is conveniently computed:

$$\text{No. fecal streptococci per 100 ml} = \frac{\text{No. fecal streptococcus colonies counted}}{\text{Sample filtration volume in ml}} \times 100$$

B Test for Members of Fecal Streptococcal Group based on KF-Agar

1 Preparation of the culture medium

a Formula: (The dehydrated formula of Bacto 0496 is shown, but equivalent constituents from other sources are acceptable). Formula is in grams per liter of reconstituted medium.

Bacto proteose peptone #3	10.0	g
Bacto yeast extract	10.0	g
Sodium chloride (reagent grade)	5.0	g
Sodium glycerphosphate	10.0	g
Maltose (CP)	20.0	g
Lactose (CP)	1.0	g
Sodium azide (Eastman)	0.4	g
Sodium carbonate (Na_2CO_3 reagent grade)	0.636	g
Brom cresol purple (water soluble)	0.015	g
Bacto agar	20.0	g

2,3,5-Triphenyl tetrazolium chloride reagent (TPTC)

This reagent is prepared by making a 1% aqueous solution of the above chemical passing it through a Seitz filter or by bringing to a boil. It can be kept in the refrigerator in a screw-capped tube until used.

b The dehydrated medium described above is prepared for laboratory use as follows:

Suspend 7.64 grams of the dehydrated medium in 100 ml of distilled water in a flask with an aluminum foil cover.

Place the flask in a boiling waterbath, melt the dehydrated medium, and leave in the boiling waterbath an additional 5 minutes.

Cool the medium to 50°-60°C, add 1.0 ml of the TPTC reagent, and mix.

For membrane filter studies, pour 5-8 ml in each 50 ml glass or plastic culture dish or enough to make a layer approximately 1/8" thick. For plate counts, pour as for standard agar plate counts.

Note: Plastic dishes containing media may be stored in a dark, cool place up to 30 days without change in productivity of the medium, provided that no dehydration occurs. Plastic dishes may be incubated in an ordinary air incubator. Glass dishes must be incubated in an atmosphere with saturated humidity.

2 Apparatus, and materials as given in Table 1.

3 General procedure is as given in I.

Special instructions

a To prepare plates open the containers, and pour 5-8 ml of sterile melted KF-Agar into each. Replace the covers of the culture containers, and allow them to stand until the agar has solidified.

b Incubate 48 hours, inverted with 100% relative humidity after filtration.

c After incubation, remove the cultures from the incubator, and count colonies under wide field binocular dissecting microscope, with magnification set at 10X or 20X. Fecal streptococcus colonies are pale pink to dark wine-color. In size they range from barely visible to approximately 2 mm in diameter. Colorless colonies are not counted.

d Report fecal streptococcus count per 100 ml of sample. This is computed as follows:

$$\text{No. fecal streptococci per 100 ml} = \frac{\text{No. fecal streptococcus colonies}}{\text{Sample filtration volume in ml}} \times 100$$

C Confirmation of Streptococcus Colonies

1 Confirmation of colony identification may be required in waters containing large numbers of Micrococcus organisms. This has been noted particularly with bathing waters, but the problem is by no means limited to such waters.

2 A confirmatory procedure is described in "Standard Methods for the Examination of Water and Wastewater, 12th ed, (1965), p 619. The worker should use this reference for the step-by-step procedure.

IV PROCEDURES FOR USE OF MEMBRANE FILTER FIELD UNITS

A Culture Media

1 The standard coliform media used with laboratory tests are used.

2 To simplify field operations, it is suggested that the medium be sent to the field, preweighed, in vials or capped culture tubes. The medium then requires only the addition of a suitable volume of distilled water-ethanol prior to sterilization.

3 Sterilization procedures in the field are the same as for laboratory methods.

4 Laboratory preparation of the media, ready for use, would be permissible provided that the required limitations on time and conditions of storage are met.

B Operation of the Sabro Field Unit

1 Equipment and materials

Sabro field unit

Membrane filters

Absorbent pads for nutrient

Culture containers

M-Endo Broth MF or L.E.S. Endo Medium

Provision for heating water (optional)

Source of electricity

2 Procedure (Based on M-Endo Broth MF)

a Connect the electric cord to the power source and to Sabro field unit. After about 15 minutes check the temperature in the incubator drawer. The desired temperature is 35°C (95°F). If the temperature is too low it can be increased by turning the thermostat adjustment screw counterclockwise. This screw is located at the front on the recessed divider between the two incubator drawers. To lower temperature, turn the adjustment screw clockwise.

b Review the supply of expendable materials to be used with the unit and secure replacements as needed (culture containers, medium, membrane filters, absorbent pads for nutrient, fuel, etc.).

c Sterilize the funnel unit by one of the following procedures.

1) Immerse the equipment 30 seconds in water at 170°F (78°C).

2) Immerse the equipment 2 minutes in boiling water (Preferred at SEC).

3) Flame-sterilize membrane filter holder inside and both ends of funnel (suggested by manufacturer).

d Lay in a row all the culture containers to be used in the filtration series, and number the containers to correspond with numbers of the data sheet.

e Place one sterile absorbent pad in each culture container. Use sterile forceps for all manipulation of absorbent pads and filters.

f Using sterile pipette deliver enough culture medium to saturate each absorbent pad. The amount of culture medium required is approximately 2 ml, but cannot be precisely stated. Sufficient medium should be applied, that when the culture container is tipped, a good-sized drop of culture medium freely drains out of the absorbent pad.

g Using sterile forceps, place a membrane filter, grid-side up, on the MF receptacle of the funnel unit. Place the funnel portion over the membrane, and clamp the unit with the spring clamp provided with the portable kit.

h Pour the water sample into the funnel. The sample can be measured in a graduated cup or cylinder previously sterilized by 2 minutes immersion in boiling water and cooled before use.

i Connect the tubing of the vacuum pump to the receptable on the base of the filter unit and draw the sample through the membrane.

j Disassemble the funnel unit and with sterile forceps transfer the membrane filter grid-side up to the appropriate culture container. The membrane should be "rolled on" the absorbent pad containing culture medium, to prevent entrapment of air between the pad and the membrane filter.

k Repeat steps g - j for additional filtrations of the same or different sampling volumes for the water being tested.

l After completion of the last filtration from any one sample, resterilize the funnel unit by one of the procedures described in instruction 2c.

m After completion of filtration, place the culture container (with membrane position grid-side down) in the incubator drawers.

n Allow the cultures to incubate 20-24 hours.

o Remove the cultures from the incubator and count coliform colonies.

C Operation of Millipore Field Monitor Unit

1 Equipment and materials

Millipore field monitor with sampling tube

Metal syringe with bypass valve

Sampling container (glass, metal, or paper measuring cup)

M-Endo Broth MF, 1.5 ×, freshly prepared or ampouled medium

Sterile pipettes

Field incubator

2 Procedure

a Remove the rubber caps from the field monitor.

b Assemble the sampling tube, field monitor, and metal syringe as follows:

Tightly insert the lateral nozzle of the syringe bypass valve in the opening of the field monitor nearest the screen side (i.e., with the membrane filter directed away from the bypass valve).

Insert the plastic adapter of the sampling tube in the opening of the field monitor nearest the membrane filter.

c Fill the sampling container with the volume of water to be tested.

If the sampling container is glass or metal it is recommended that it be previously sterilized by 2 minutes immersion in boiling water, then cooled before use.

Paper measuring cups, if preferred, should be rinsed repeatedly in the water being tested, before the actual sample is filtered.

d Insert the sampling tube into the cup and draw out all the water by pumping the syringe. Filtrate water is expelled from the end of the bypass valve in this procedure. Caution is required to direct this syringe in such a way that expelled water does not create a mess.

e Invert the syringe (membrane side up) and draw the remaining water through the filter.

f Apply culture medium as follows:

Remove the sampling tube from the field monitor.

Apply freshly prepared 1.5x M-Endo Broth MF with a sterile dropping pipette (sterilize by 2 minutes immersion in boiling water). The tip of the pipette should be introduced into the opening of the field monitor away from the membrane filter; the medium should be delivered by a series of short pulsing contractions of the pipette bulb. Alternately, if ampouled medium is used, break the tips off an ampoule, and use the ampoule like a pipet in delivering culture medium to the absorbent pad beneath the membrane filter in the assembly.

g Remove the monitor from the syringe, replace the rubber caps, and place inverted in the field incubator.

h Incubate the cultures 22-24 hours, then remove from the incubator and count coliform and noncoliform colonies in the usual way.

D Operation of Water Testing Kit, Bacteriological (This is the Millipore kit developed for military use, now commercially available)

1 Supporting supplies and equipment are the same as for the laboratory procedures.

2 Set the incubator voltage selector switch to the voltage of the available supply, turn on the unit and adjust as necessary to establish operating incubator temperature at 35 $\pm 0.5^{o}C$.

3 Sterilize the funnel unit assembly by exposure to formaldehyde or by immersion in boiling water. If a laboratory autoclave is available, this is preferred.

Formaldehyde is produced by soaking an asbestos ring (in the funnel base) with methanol, igniting, and after a few seconds of burning, closing the unit by placing the stainless steel flask over the funnel and base. This results in incomplete combustion of the methanol, whereby formaldehyde is produced. Leave the unit closed for 15 minutes to allow adequate exposure to formaldehyde.

4 Filtration and incubation procedures correspond with laboratory methods.

5 The unit is supplied with a booklet containing detailed step-by-step operational procedures. The worker using the equipment should become completely versed in its contents and application.

E Counting of Colonies on Membrane Filters

1 Equipment and materials

Membrane filter cultures to be examined

Illumination source

Simple lens, 2X to 6X magnification

Hand tally (optional)

2 Procedure

a Remove the cultures from the incubator and arrange them in numerical sequence.

b Set up illumination source as that light will originate from an area perpendicular to the plane of membrane filters being examined. A small fluorescent lamp is ideal for the purpose. It is highly desirable that a simple lens be attached to the light source.

c Examine results. Count all coliform and noncoliform colonies. Coliform colonies are pink to deep red and have a "metallic" surface sheen under reflected light, which may cover the entire colony or may appear only on the center.

Noncoliform colonies range from colorless to pink or red, but do not have the characteristic "metallic" sheen.

d Enter the colony counts in the data sheets.

e Enter the coliform count per 100 ml of sample for each membrane having a countable number of coliform colonies. Computation is as follows:

No. coliform per 100 ml =

$$\frac{\text{No. coliform colonies on MF}}{\text{No. milliliters sample filtered}} \times 100$$

REFERENCES

1 Standard Methods for the Examination of Water and Wastewater. APHA, AWWA, WPCF. 12th Edition. 1965.

2 McCarthy, J. A., Delaney, J. E., and Grasso, R. J. Measuring Coliforms in Water. Water and Sewage Works. 1961: R-426-31. 1961.

This outline was prepared by H. L. Jeter, Director, Training Branch, Ohio Basin Region, FWPCA.

SELECTION OF SAMPLE FILTRATION VOLUMES FOR MEMBRANE FILTER METHODS

I INTRODUCTION

A Wide Range of Filtration Volumes

1 The membrane filter permits testing a wide range of sample volumes, from several hundred milliliters to as little as 0.0001 ml, or even less. Suitable dilution of sample volumes smaller than 1.0 ml may be required for accuracy of sample measurement.

2 While the method lends itself to a wide range of sample volumes, the filter has limitations in the number of isolated (or countable) differentiated colonies which can develop on the available surface area. Figure 1 illustrates a common pattern of colony counts over a wide range of sample filtration volumes.

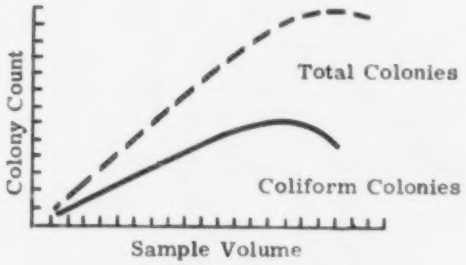

Figure 1

a The graph is based on coliform determinations using M Endo Broth MF. The line designated "Total Colonies" includes both coliform and noncoliform colonies. The "Coliform Colonies" line refers only to differentiated colonies having the typical color and sheen of coliform colonies on the medium.

b For both the total colonies and the coliform colonies there is a proportional relationship between colony count and sample volume over much of the range of sample volumes. With increasing colony counts, there are some levels above which the proportional relationship fails, both for total and for coliform colonies.

c In the straight lines in Figure 1, where there is proportionality between colony counts and filtration volumes, it is possible to compute density of bacteria in the sample, based on the equation:

$$\text{No. organisms per 100 ml} = 100 \times \frac{\text{No. colonies counted}}{\text{No. ml of sample filtered}}$$

The equation is not quantitatively reliable in the curved portions of the lines.

B Scope of this Presentation

1 To explain limitations on quantitatively reliable colony counts on membrane filters.

2 To present numbers of colonies acceptable for quantitative tests with available media.

3 To demonstrate the different quantitative bacterial density ranges determined by single-volume filtrations, with available media.

4 To demonstrate quantitative ranges covered by a series of filtration volumes with currently-used differential media.

5 To provide guidance for selection of sample filtration volumes under the following practical conditions:

a When there is need to determine compliance with established bacteriological water quality standards.

b When there is need to determine density of a specified bacterial group.

1) In the absence of prior bacteriological data, and

2) When prior bacteriological data are available.

II LIMITATIONS ON COLONY COUNTS USED FOR QUANTITATIVE WORK

A Bacterial Density

1 The minimum sample volume should result in production of at least 20 colonies of the bacteria being counted. Sample volumes yielding lesser numbers of colonies are subject to unacceptably large random variations in the computed bacterial density, determined as above (I, A, 2).

2 The maximum acceptable colony density, for quantitative determinations, is variable with the bacterial group tested and the medium used. Factors influencing maximum acceptable colony density include:

a Size of colonies. In principle, each colony should represent one bacterial cell deposited on the filter, or, conversely, each bacterial cell deposited on the filter should result in production of a recognizable colony. Media producing relatively large colonies (as in the fecal coliform test) will support smaller numbers of colonies on the filter than media producing smaller colonies (such as fecal streptococci). If the colony size is large and the number of bacteria deposited on the filter is great, some colonies will represent two or more cells initially deposited on the filter, and the quantitative reliability of the test is impaired.

b Selectivity of medium. Highly selective media permit growth of relatively few colonies of extraneous, unwanted, bacteria. The available area of the filter is occupied primarily by colonies of the group tested. Thus, with a highly selective medium such as that used for fecal streptococci, it is reasonable to expect good quantitative results with relatively high colony counts. Conversely, media having limited selectivity (such as Endo-type media for coliforms) supports growth of considerable numbers of extraneous bacterial colonies, and it is necessary to place arbitrary limitations on the number of colonies per membrane in quantitative studies.

c Biochemical interference between neighboring colonies. Associated with the physical crowding effects noted in (b) above, sheen production of coliform colonies may be inhibited by overcrowding of colonies. This reinforces the need for restriction of colony density on the filter.

B Suspended Matter

1 Particulate matter in the sample can be a limitation in application of membrane filters, especially when the amount of suspended matter is relatively great and the bacterial density is low.

2 Difficulties from suspended matter in the sample may be apparent in several ways.

a The pores of the filter may be occluded, limiting the volume of sample that can be filtered. This problem has been noted in waters rich in clays and in waters containing large populations of certain diatoms or other algae.

b Fibrous matter can be troublesome, due to the tendency for a capillary film of liquid culture medium to form around the fibers. Colonies in contact with such fibers tend to grow along the path of the fibers, assuming highly irregular forms. Sometimes these colonies cover abnormally large areas of the filter surface.

c A more or less continuous mat of particles may be collected from some samples, with each particle soon surrounded by a film of liquid culture medium. On such filters, distinct colonies usually fail to develop as discrete entities, but grow in a more or less continuous film over the entire surface of the filter.

3 Problems due to particulate matter often can be reduced by filtration of the selected volume of water in two or more smaller increments, through separate filters. In effect, this is a means of enlarging the available surface area of the filter.

Prefiltration of the sample through a coarse filter for preliminary removal of extraneous particulate matter is not recommended in quantitative work. Prefiltration invariably results in removal of unpredictably large numbers of bacterial cells.

In some cases the problem of particulates cannot be solved, and it must then be conceded that the membrane filter method is not acceptable for such samples. It then becomes necessary to resort to other procedures, such as the dilution tube method or agar plating methods.

III LIMITS ON NUMBER OF COLONIES ON FILTERS WITH VARIOUS MEDIA

Referring to Figure 1, a specific number of colonies is not shown for acceptable proportionality between colony number and filtration volume. Fixed limits cannot be stated for all test situations, for these limits are somewhat variable from one culture medium to another and from one sample source to another.

The recommended limits shown in Table 1 are empirical values based on research experience. It is believed that quantitative determinations of acceptable statistical reliability can be obtained if the determinations are based on colony counts within the limitations shown.

IV RANGE OF BACTERIAL DENSITIES COVERED BY SINGLE-VOLUME FILTRATIONS

A The equation used in Section I of this outline can be used with any sample filtration volume to determine the bacterial density range over which acceptable counts can be made.

For example, assume that a sample of 10 ml is used for a quantitative determination of total coliforms. Based on Table 1, quantitative determinations should be based on a filtration volume yielding 20 - 80 coliform colonies. Compute the coliforms per 100 ml based on 20 colonies and on 80 colonies per filter. This will be the bacterial density range covered by a 10 ml filtration volume, thus:

for 20 colonies:

$$\text{No. coliforms per 100 ml} = 100 \times \frac{20}{10} = 200$$

and for 80 colonies,

$$\text{No. coliforms per 100 ml} = 100 \times \frac{80}{10} = 800$$

Thus, a 10 ml sample portion is appropriate for determination of total coliforms in the range 200 - 800 per 100 ml.

B Table 2 illustrates the ranges covered for several filtration volumes, with colony counts in the ranges 20 - 60, 20 - 80, and 20 - 100 per filtration volume.

Table 1. RECOMMENDED COLONY COUNT RANGES FOR QUANTITATIVE DETERMINATIONS WITH MEMBRANE FILTER TESTS

Test	No. colonies		Medium	Remarks
	Minimum	Maximum		
Total Coliform	20	80	M Endo Broth MF, LES Endo Medium	Not more than 200 colonies of all types
Fecal Coliform	20	60	M FC Broth	
Fecal Streptococci	20	100	M Enterococcus Agar, KF Agar	
Total Counts	20	200	M Enrichment Broth	Spreaders may require adjustment

Table 2. RANGES COVERED BY REPRESENTATIVE FILTRATION VOLUMES

Ml sample filtered	Bacterial count per 100 ml based on			
	20 colonies	60 colonies	80 colonies	100 colonies
100	20	60	80	100
10	200	600	800	1000
1	2000	6000	8000	10, 000
0. 1	20,000	60, 000	80, 000	100, 000
0. 01	200, 000	600, 000	800, 000	1, 000, 000

C Application of a Series of Filtration Volumes

1 Examination of Table 2 shows that for quantitative work on membrane filters, to extend the range of any test, it is necessary to filter two or more different sample volumes. The worker uses the one sample volume yielding a quantitatively acceptable number of colonies to compute the bacterial count per 100 ml.

2 Further, it can be seen that varying the filtration volumes by decimal increments will be inappropriate; there are values within the total range covered in which the colony number would fall outside the critical counting range for the test being made.

3 In order to give maximum assurance that a series of varying filtration volumes will yield at least one membrane with an acceptable number of colonies, the range of filtration volumes should be along these lines:

a Total coliform counts should be based on filtration volumes varying by a factor of 4, or less.

b Fecal coliform counts should be based on filtration volumes varying by a factor of 3, or less.

c Fecal streptococcus counts should be based on filtration volumes varying by a factor of 5, or less.

V SELECTING FILTRATION VOLUMES FOR MEMBRANE FILTER TESTS

A Total Coliform Counts

1 Determination of compliance with existing bacterial quality standards.

a For all tests to determine whether water meets PHS Drinking Water quality standards, minimum sample sizes are prescribed as 50 ml, with 100 ml sample volumes suggested.

b With tests in which it is assumed that coliforms are present in some numbers, and the test is to determine whether some limiting standard (as 1000 per 100 ml in natural bathing waters, prescribed by some agencies), another approach is suggested. Here, select the sample filtration volume which would be quantitatively most acceptable to count coliforms at the limiting value. For example, with a limiting value of 1000 per 100 ml:

$$\text{No. organisms per 100 ml} = 100 \times \frac{\text{No. colonies counted}}{\text{No. ml of sample filtered}}$$

This previously given equation can be rearranged to:

$$\text{Sample filtration volume in ml} = 100 \times \frac{\text{No. colonies counted}}{\text{No. organisms/100 ml}}$$

and from this:

$$\text{Sample filtration volume in ml} = 100 \times \frac{50}{1000}$$

$$= 5$$

(The value 50 is the midrange number of colonies for an acceptable colony count of 20 - 80 for computing coliforms per 100 ml)

2 In quantitative work, to determine number of coliforms per 100 ml the worker may or may not have prior information or standards to use as guidance in selecting filtration volumes.

a In absence of prior bacteriological data

1) Unpolluted raw surface water, 1, 4, 15, and 60 ml samples will cover a count range of 33 - 8000 per 100 ml.

2) Polluted raw surface water, 0.02, 0.08, 0.15, and 0.5 ml samples will cover a count range of 4000 to 40,000 per 100 ml.

3) Sewage and dilute sewage, with filtration volumes of 0.0003, 0.001, 0.003, and 0.01, will cover a count range of 200,000 to 27,000,000 per 100 ml.

b If prior coliform data are available

Use the equation:

$$\text{Basic filtration volume in ml} = 100 \times \frac{50}{\text{Average coliform count}}$$

Example: Assume that prior data indicate average coliform count of 35,000 per 100 ml. Using the equation:

$$\text{Basic filtration volume in ml} = 100 \times \frac{50}{35{,}000}$$

$$= 0.143 \text{ ml}$$

Round off the filtration volume to 0.15 ml.

To assure a reasonable count-range, filter increments of 0.04 and 0.60 ml in addition. This will provide for acceptable coliform counts in the range of 3300 to 200,000 per 100 ml.

B Fecal Coliform Counts

1 Currently, no water standards are based on fecal coliform organisms.

2 Determination of fecal coliforms in the absence of prior data.

a Unpolluted raw surface water: Filter 1, 3, 10, and 30 ml sample portions. These volumes will cover a fecal coliform range of 67 - 6000 per 100 ml.

b Polluted raw surface water: Filter portions of 0.1, 0.3, 1.0, and 3.0 ml. This will cover a fecal coliform count range of 670 to 60,000 per 100 ml.

c Sewage and dilute sewage: Filter sample portions of 0.0003, 0.001 and 0.003 ml. This will provide for counts of 670,000 to 20,000,000 per 100 ml.

3 Determination of fecal coliforms in presence of prior data

a When previous fecal coliform counts are available:

$$\text{Filtration volume in ml} = 100 \times \frac{40}{\text{Av. fecal coliform count per 100 ml}}$$

Example: Prior data show 8000 fecal coliforms per 100 ml.

$$\text{Basic filtration volume in ml} = 100 \times \frac{40}{8000} = 0.5$$

Filter volumes of 0.15, 0.5 and 1.5 ml. This will be suitable for fecal coliform counts over the range 1300 to 40,000.

b When previous total coliform data are available but no fecal coliform data are available, use the total coliform value as above, but filter 3x and 9x the computed basic volume.

Example (from above): Computed basic value = 0.5 ml

Filter volumes of 0.5, 1.5 and 5.0 ml.

C Fecal Streptococcus Determinations

1 In absence of prior data

a Unpolluted raw surface water: Filter sample portions of 1, 5, 25 and 100 ml. This will provide for fecal streptococcus counts in the range 20 to 10,000 per 100 ml.

b Polluted surface water: Filter sample portions of 0.1, 0.5, and 2.0 ml. This will provide for fecal streptococcus counts in the range 1000 to 100,000 per 100 ml. Provision for rather high counts of fecal streptococci is made because of possible situations in which pollution of the water originates from domestic or wild animals. In the event that such pollution is highly improbable, a filtration series of 0.2, 1.0, and 5.0 ml (covering a count range of 400 to 50,000 per 100 ml) would be more appropriate.

2 When prior data are available

a If coliform, but not fecal streptococcus data are available, compute a basic filtration volume as in A, 2 above, but use the average coliform count as a point of reference. If significant pollution from domestic or wild animals is believed present, filter 0.2X, 1X and 5X the basic filtration volumes. If the pollution levels are believed due primarily to human sources, use 1X, 5X and 25X the basic filtration volumes.

b If prior streptococcus data are available, use the equation

$$\text{Basic filtration volume in ml} = 100 \times \frac{60}{\text{Av. Streptococcus count per 100 ml}}$$

and filter 0.2X, 1X, and 5X the basic filtration volume for streptococci.

REFERENCES

1 The Federal Register. October 23, 1956, pp 8110-11; and March 1, 1957. p 1271.

2 Clark, H.F., Kabler, P.W., and Geldreich, E.E. Advantages and Limitations of the Membrane Filter Procedure. Water and Sewage Works. September 1957.

This outline was prepared by H. L. Jeter, Director, Training Branch, Ohio Basin Region, FWPCA.

Exercise

WATER SUITABILITY TEST

O.W. Kaufmann*

I EQUIPMENT AND MATERIALS

A Rinse all glassware in freshly redistilled water from a glass still.

B Sterilized reagents are not employed, but every effort must be made to eliminate contamination; use clean, dry-heat sterilized borosilicate glassware covered with aluminum foil.

C Fresh redistilled water (A), boil 1 min.

D Unknown distilled water (B), boil 1 min.

E Toxic Water (F), boil 1 min.

F Dilution blanks - 99 ml empty (3)

G Aluminum foil, 2×2 in.

H Pipettes 11 ml; 10 ml; 1.1 ml.

I A. aerogenes (IMViC - - + +) Nutrient agar slant in 125 × 16 mm tube, slant length 2.5 in.

J Petri plates - 27

K SMA - 260 ml

L Dilution blanks - 99 ml buffer (8)

M Reagents, highest purity (500 ml of each)

1 Sodium citrate solution - Dissolve 0.29 g of sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$ in 500 ml of redistilled water which has been boiled for 1 min. or sterilized by membrane filtration.

2 Ammonium sulfate solution - Dissolve 0.60 g ammonium sulfate, $(NH_4)_2SO_4$, in 500 ml redistilled water which has been boiled 1 min. or filter sterilized.

3 Salt mixture solution - Dissolve 0.26 g magnesium sulfate, $MgSO_4 \cdot 7H_2O$; 0.17 g calcium chloride, $CaCl_2 \cdot 2H_2O$; 0.23 g ferrous sulfate, $FeSO_4 \cdot 7H_2O$; and 2.50 g sodium chloride,

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NaCl, in 500 ml redistilled water. Use boiled or filter sterilized water. Do not heat the solution as heat decomposes the iron salt. Store in the dark at 5°C, because sunlight converts the ferrous to ferric salt. Turbidity may appear in 3-5 days. If slight, turbidity does not interfere.

4 Stock phosphate buffer diluted 1:25. Boil 1 min. or filter sterilize. Prepare stock by dissolving 34.0 gm of potassium dihydrogen phosphate, KH_2PO_4, in 500 ml of distilled water; adjust to pH 7.2 with 1 N NaOH and dilute to 1 liter with distilled water.

5 Unless contaminated, solutions may be held for as long as 2 weeks if stored at 5°C.

II PROCEDURE

A Preparation of Inoculum

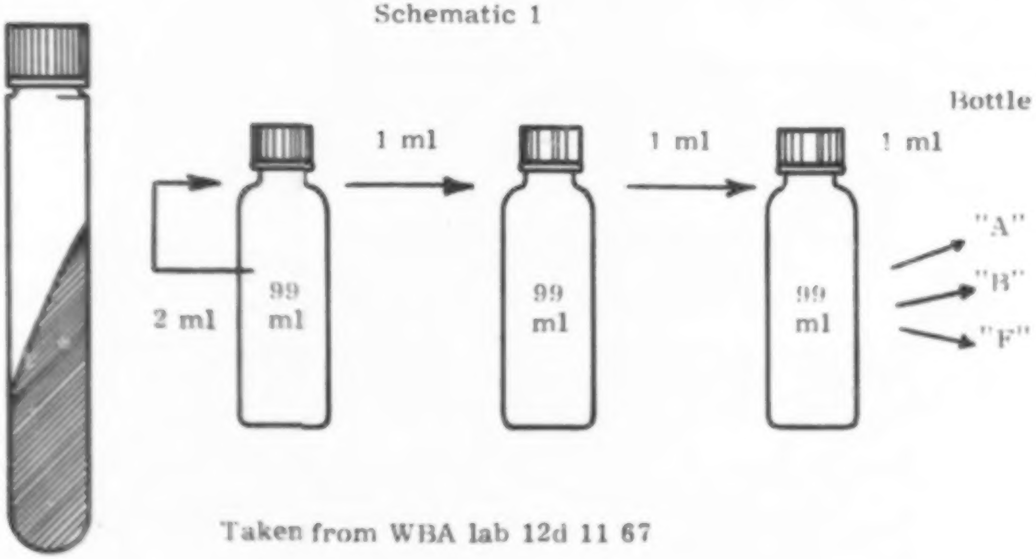
1 Upon removing culture from refrigerator, subculture twice at 24-hr. intervals to NA slants; inoculate so as to cover the surface completely. (Example - start the first subculture on Friday (AM) and incubate it at 32 or 35°C until Monday; make a transfer from this 72-hr. old culture, and incubate it for 24 hours; this 24-hr. old culture is used on Tuesday to set up the test).

2 To the second subculture add 2 ml of phosphate buffer from a 99 ml blank, and shake the tube vigorously for about 45 sec. to remove growth completely. Scrape, if necessary.

3 Return the suspension to the original dilution blank; allow to drain for 30 sec., and touch off the drop, if present. Shake this 1:100 dilution in the standard manner.

4 Prepare a 10^{-6} dilution in the usual manner. Use 0.1 ml of the 10^{-6} dilution to inoculate each test sample. (See Schematic 1)

Schematic 1



Taken from WBA lab 12d 11 67

B Preparation of Synthetic Medium

1 Label three empty dilution bottles "A", "B" and "F", and add solutions as indicated in the protocol below:

	Synthetic Media		
Reagents	"A"	"B"	"F"
Citrate Sol.	2.5	2.5	2.5
Ammonium Sol.	2.5	2.5	2.5
Salt Sol.	2.5	2.5	2.5
Phosphate Buffer (1:25)	1.5	1.5	1.5
Redistilled Water (A)	21.0	---	---
Unknown Water (B)	---	21.0	---
Toxic Water (F)	---	---	21.0

2 Inoculate each bottle with 0.1 ml of the diluted A. aerogenes culture.

3 Swirl to thoroughly mix, and plate 1.0 ml from each bottle to determine the initial number per ml. (The initial count should be from 25-75 per ml).

4 Plate 1.0 ml of each reagent to detect contamination.

5 Incubate all plates at 32°C for 20-24 hrs.

6 Incubate bottles ("A", "B" and "F") at 32° or 35°C for 20-24 hrs.

7 After 24 hrs. incubation shake all test samples and prepare a 10^{-2} and 10^{-4} dilution of each. Plate 1.0 ml and 0.1 ml from bottle "A", "B", and "F" dilution.

8 Incubate plates at 32°C for 20-24 hrs. and count.

9 Record all data in the following table and determine the suitability of the unknown distilled water sample (B) and (F.)

C Interpretation of Results

1 A ratio [B/A] between 0.8 to 1.2 indicates that no toxic substances are present in sample. A ratio [B/A] as high as 3.0 would have little significance in actual practice; the 1.2 ratio could go as high as 3.0 without any undesirable results.

2 A ratio [B/A] less than 0.8 indicates that growth inhibiting substances are present in the sample. A similar interpretation can be applied to the F/A ratio.

Sample Description	No. of colonies on each plate					Ratio B/A*
	1.0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	
A-after 24 hrs. inc.						---
B-after 24 hrs. inc.						
F-after 24 hrs. inc.						
Controls						
Citrate sol. a		---	---	---	---	---
Amm. sol. b		---	---	---	---	---
Salt sol. c		---	---	---	---	---
Buffer sol. d		---	---	---	---	---
Redist. H_2O, A		---	---	---	---	---
Dist. H_2O, B		---	---	---	---	---
Toxic H_2O, F		---	---	---	---	---
Init. no. in A		---	---	---	---	---
Init. no. in B		---	---	---	---	---
Init. no. in F		---	---	---	---	---

*Convert all values to no./ml before calc. ratio.

10-1

INTRODUCTION TO STATISTICS

Part I

Summarization of Data

J.H. Parker*

I INTRODUCTION

The body of statistical methods comprises two groups generally called descriptive statistics and statistical inference. Descriptive statistics encompasses primarily graphical techniques for the presentation of data while statistical inference is basically a mathematical approach to the problem of inferring from a part to the whole.

II BASIC GRAPHICAL TECHNIQUES

In this section we consider the non-mathematical techniques of summarizing a set of data in order that the meaningful information can be extracted from it.

Consider the data in Table 1 which represent aqueous fluoride ion concentrations determined by a colorimetric procedure.

Table 1

F^-, mg/liter		
0.68	0.77	0.85
0.71	0.79	0.85
0.72	0.80	0.87
0.74	0.81	0.90
0.75	0.82	0.91
0.77	0.83	0.95

A Frequency Table

As the first step in summarizing a set of data we form a frequency table. (See Table 2). To construct the table the data are divided into a number of intervals of equal length called class intervals and the number of results falling into each interval is recorded in the "frequency" column. The number of class intervals choosen is arbitrary. However, it is a rule of thumb to choose the length of the class interval so that 7 to 15 intervals will include all the data under consideration.

Table 2

Class Interval	Frequency
.65 au* .70	1
.70 au .75	3
.75 au .80	4
.80 au .85	4
.85 au .90	3
.90 au .95	2
.95 au 1.00	1

*and under

B Frequency Polygon

As a further step we can graph the frequencies recorded in the frequency table. One way of doing this is to plot the frequency along the ordinate (vertical axis) and the midpoint of the class interval along the abscissa (horizontal axis). The line connecting the plotted points in Figure 1. forms a frequency polygon.

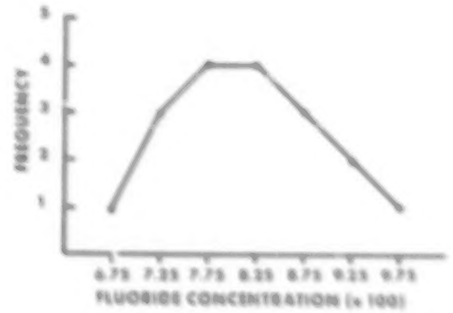


Figure 1

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C Histogram

Another method of graphing the frequency information is to construct a histogram as shown in Figure 2. The histogram is a two dimensional graph in which the length of the class interval is taken into consideration. Each class interval becomes the base of a rectangular bar whose height is equal to the corresponding frequency.

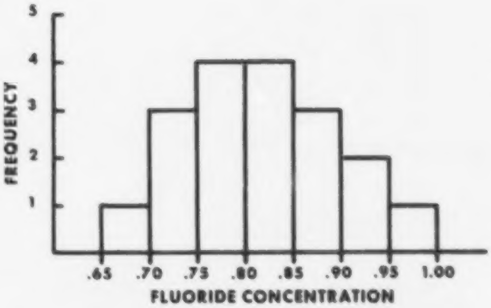

Figure 2

D Histogram of Relative Frequency

The histogram can be a very useful tool in statistics especially if we convert the given frequency scale to a relative scale such that the sum of all the ordinates equals one. Thus, each ordinate value is derived by dividing the original frequency by the number of observations in the sample. For the data in Table 1 we would divide each of the frequency values in Table 2 by 18.

The advantage of constructing a relative frequency histogram like the one in Figure 3 is that we can interpret areas under the histogram as probabilities provided we assume a scale on the abscissa such that each class interval is of unit length. Then the probability that a given value will fall in any one interval is the area under the histogram in that interval. For example, the probability that a value will fall between .70 and .75 is equal to the area under the histogram in that interval which is $3/18 \times 1 = 1/6$.

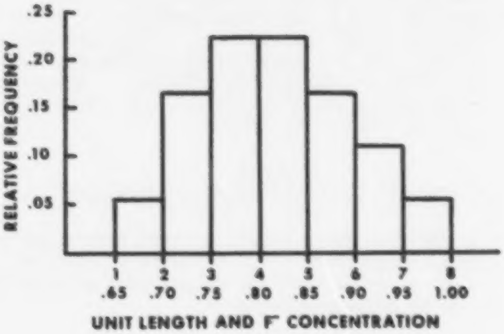

Figure 3

E Cumulative Frequency Distribution

From a frequency table (See Table 2) we can construct a cumulative frequency table as shown in Table 3. The cumulative frequency table gives the number of observations less than a given value. The cumulative frequency values in Table 3 are found by summing the frequencies in Table 2.

Table 3

F^- conc.	Cum.Frequency
under .70	1
" .75	4
" .80	8
" .85	12
" .90	15
" .95	17
" 1.00	18

10-3

If we convert the cumulative frequency values to relative frequencies as we did to construct the relative frequency histogram, we can plot the relative values to form a cumulative frequency curve as shown in Figure 4. The probability that a result will fall below a given value can be read from the cumulative frequency curve. For example, to find the probability that a result will be less than 0.85 mg/liter we read up to the curve at the point x = .85 and across to the value .67 on the probability axis.

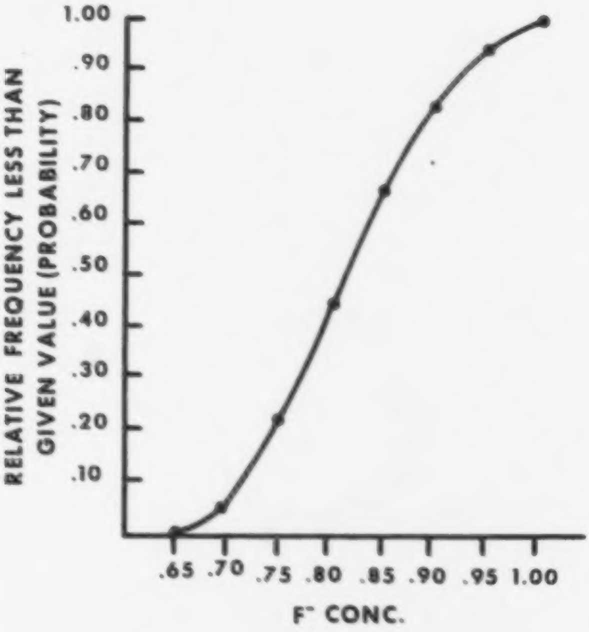

Figure 4

INTRODUCTION TO STATISTICS

PART II

John H. Parker*

MEASURES OF CENTRAL TENDENCY AND DISPERSION

I INTRODUCTION

From a practical point of view, statistical inference is the application of mathematical tools to analyze and interpret the results of investigations in the physical and social sciences with respect to the hypotheses under investigation. The need for a statistical approach rests on the well-founded assumption that any such result contains an inherent component of error or random variation which introduces uncertainty into any conclusions which may be drawn from the observed results.

A Mathematical Distributions

It is further assumed that the error components can be described by a mathematical function called a probability distribution. Knowledge of the relevant type of theoretical distribution provides us with the means of assessing the uncertainty (risk of being incorrect) in conclusions drawn from observed results.

B Population Parameters

The concept of distribution pertains to the totality of possible results which would be observed in an infinite number of repetitions of an experiment or investigation. This theoretically infinite number of results is called a population. Any measurable characteristic of a population distribution is called a parameter. For each type of distribution there exists a characteristic set of parameters. Specific values for the parameters of a given distributional form define a particular distribution of that type. In almost every case the entire population can never be observed and therefore the values for the parameters are unknown. Population parameters are denoted by lower case Greek letters.

C Sample Statistics

The observed results from a particular investigation are called a sample of the relevant population. In almost every case the sample is a subset of the population.

A statistic is a function of the sample points (i.e., observed results) which usually estimates a population parameter. Sample statistics are denoted by English letters.

D Classification of Statistical Measures

It is implied above that the type of population distribution determines the particular function of the observed results (i.e., sample statistic) which will provide the "best" estimate of the corresponding population parameter. For this reason it is advantageous to assume that any given sample came from a population with a specified form of distribution. The use of graphical techniques to indicate the form of distribution should now be apparent.

In most situations the distributions or frequency functions of interest have a single peak and characteristic dispersion of area about this peak. Parameters which locate the distribution (by the peak, center of gravity, midpoint, etc.) are called location parameters and the corresponding sample statistics are classified as measures of central tendency. Parameters which describe the dispersion of area are called scale parameters and the corresponding sample statistics are classifed as measures of dispersion. Ideally, one would like to encounter only distributions for which one measure of central tendency and one measure of dispersion summarize all of the relevant information in the sample about the population.

II MEASURES OF CENTRAL TENDENCY

In this section and the next we define and lay out the computational format for the more commonly used measures in statistics. We let x_i denote a typical observed result so that $[x_1, x_2, \ldots, x_n]$ represents a sample of n observations.

A The Mean

The most commonly used measure of central tendency is the mean or arithmetic average. We denote the sample mean by $\bar{x}$ and the population mean, of which $\bar{x}$ is terms that we will have when using a mean that has been estimated from the sample. In line with above, we should draw the distinction between the variance of the sample and the sample variance. The variance of the sample, we would calculate for its own sake and divide by n. However, we calculate the sample variance as an estimate of the population variance and divide by n - 1. There is no practical use of the variance of the sample, therefore, we always calculate the sample variance dividing by n - 1 which we shall call the number of degrees of freedom in our sample. As a rule, in any calculation, for every parameter that must be estimated, one degree of freedom (d. f.) is lost.

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C The Range

The range is also used as a measure of dispersion. It is the difference between the highest and lowest values in a set of data.

$$R = \max(x_i) - \min(x_i).$$

For the data in Table 1, the range is then:

$$R = 0.95 - 0.68 = 0.27.$$

A rough estimate of s can be made by dividing the range of the sample by the square root of n, the number of observations, when n is small.

$$s \simeq \frac{0.27}{\sqrt{18}} \simeq \frac{0.27}{4} \simeq 0.67$$

The use of the range is limited to instances where the labor of computing the standard deviation is impractical.

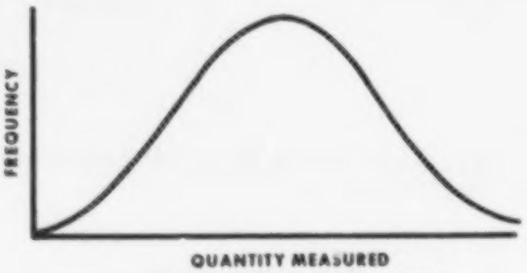
IV THE NORMAL DISTRIBUTION

A The most important theoretical distribution in statistics is the familiar bell-shaped normal distribution which is symmetric about its peak (See Figure 1.). The following assumptions give rise to this distributional form:

1 Values above or below the mean are equally likely to occur.

2 Small deviations from the mean are extremely likely.

3 Large deviations from the mean are extremely unlikely.



NORMAL DISTRIBUTION CURVE

Figure 1.

The normal distribution is completely defined by its mean, μ, and its standard deviation σ in the following manner:

1 The area under the normal curve between μ minus σ and μ plus σ is 68 percent of the total area, to the nearest 1 percent.

2 The area under the normal curve between μ minus 2σ and μ plus 2σ is 95 percent of the total area, to the nearest 1 percent.

3 The area under the normal curve between μ minus 3σ and μ plus 3σ is 99.7 percent of the total area, to the nearest 0.1 percent.

If a frequency curve is a good approximation to the normal curve, these characteristics of the normal curve can be used to five information about the frequency distribution.

V TRANSFORMATION OF DATA

A Skewed Distribution

In some areas of investigation one often encounters distributions which are not symmetric. For example, distributions of bacterial counts are often characterized by many more extremely high counts relative to the median than extremely low counts. The frequency curves of these distributions have a long right tail as shown in Figure 2. Distributions of this type display positive skewness.

B Logarithmic Transformation

For many reasons, both practical and theoretical, we prefer to work with symmetric distributions like the normal curve. Therefore, it is desirable to transform skewed an estimate, by μ. The computational formula is:

$$\overline{x} = \frac{\Sigma x_i}{n}.$$

For the fluoride data in Table 1. we calculate the sample mean as follows:

$$\overline{x} = \frac{\Sigma x_i}{n} = \frac{14.52}{18} = 0.807$$

Table 1.

F^-, mg/liter		
0.68	0.77	0.85
0.71	0.79	0.85
0.72	0.80	0.87
0.74	0.81	0.90
0.75	0.82	0.91
0.77	0.83	0.95

B The Median

Another common measure of central tendency is the median. The median is the value such that half of the other values are greater and half are less. To find the sample median we need only arrange the data in ascending and descending order and pick the middle value. When there is an even number of observations take the average of the two middle values. For the data in Table 1. the medain is 0.805.

C The Mode

The mode is another measure of central tendency, although it is of little practical importance. The mode is the most frequently occurring value. Therefore, the population mode is the value corresponding to the peak of the frequency distribution curve. Frequency distributions with more than one peak are called multimodal. In a symmetrical frequency curve the mean, median, and mode are all equal.

III MEASURES OF DISPERSION

A The Standard Deviation

As with measures of central tendency, there are several measures of dispersion, the most common of which is the standard deviation. We denote the sample by s and the population value of which s is an estimate by σ. The computational formula is:

$$s = \sqrt{\frac{\Sigma (X_i - \overline{X})^2}{n - 1}}$$

However, computation using this formula is tedious and it is relatively simple to show the following relationship:

$$s = \sqrt{\frac{\Sigma (X_i - \overline{X})^2}{n - 1}} = \sqrt{\frac{n \Sigma X_i^2 - (\Sigma X_i)^2}{n(n - 1)}}$$

The derived formula is preferrable because of its adaptability to the desk calculator. We calculate the sample standard deviation of the fluoride data in Table 1. as follows:

$$s = \sqrt{\frac{(18)(11.8048) - (14.52)^2}{18(17)}}$$

$$= \frac{212.4864 - 210\ 8304}{306}$$

$$= \frac{1.656}{306} = 0.0054 = 0.073$$

B The Variance

The sample value s^2 is referred to as the sample variance and is merely the square of the standard deviation. Often it is more convenient in conversation as well as computation to refer to the variance. This should not cause confusion if the above relation is kept in mind.

The population variance is represented by σ^2. Its formula is:

$$\sigma^2 = \frac{\Sigma (X_i - \mu)^2}{n}$$

This is the same as the formula for s^2 except we use the true population mean μ rather than its estimate $\overline{x}$ and divide by n instead n - 1.

Obviously in calculating the sample variance the true mean is not known and we, therefore, use the estimate of the mean from the data. In calculating the sample mean and then using it to calculate the variance of the same data we lose what is called a degree of freedom. It can be shown that the estimate of the variance must be based upon the sum of independent squared terms. We average our values over n - 1 because this is the number of independent squared

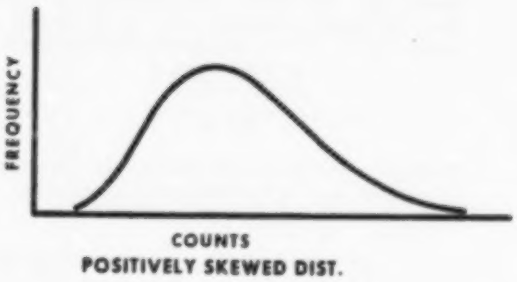


Figure 2

data in such a way that a symmetric distribution results, resembling the normal. One way of deriving an approximately normal distribution from a positively skewed distribution is by expressing the original data in terms of logarithms. An artificial sample of coliform counts and their logarithms are shown in Table 2. Comparison of the frequency tables for the original data and the logs (Table 3 and Table 4, respectively) clearly shows that the logarithms more closely approximate a symmetric distribution.

Table 2.

coliform	MPN/100 ml
MPN	log MPN
11	1.041
27	1.431
36	1.556
48	1.681
80	1.903
85	1.929
120	2.080
130	2.114
136	2.134
161	2.207
317	2.501
601	2.779
760	2.881
1020	3.009
3100	3.491

Table 3

Class Interval	Frequency (MPN)
0 au 400	11
400 au 800	2
800 au 1200	1
1200 au 1600	0
1600 au 2000	0
2000 au 2400	0
2400 au 2800	0
2800 au 3200	1

Table 4

Class Interval	Frequency(logMPN)
1.000 au 1.300	1
1.300 au 1.600	2
1.600 au 1.900	1
1.900 au 2.200	5
2.200 au 2.500	1
2.500 au 2.800	2
2.800 au 3.100	2
3.100 au 3.400	0
3.400 au 3.700	1

C Measures of Central Tendency of Skewed Distributions

If the logarithms of data from a positively skewed distribution are approximately normally distributed, we say that the original data have a log-normal distribution. The best estimate of central tendency of log-normal data is the geometric mean defined as:

$$\bar{x}_g = \sqrt[n]{(x_1)(x_2)\ldots(x_n)}\ .$$

Notice that

$$\log \bar{x}_g = \frac{\Sigma(\log x_i)}{n}$$

so that the geometric mean of the original data is equal to the antilog of the arithmetic mean of the logarithms. It is of interest that the population geometric mean is equal to the population median. Thus, in the case of data from a skewed distribution, the sample median is a better estimate of central tendency than the arithmetic mean of the original data. For the coliform data in Table 2 we calculate:

$$\log \bar{x}_g = \frac{\Sigma(\log x_i)}{n} = \frac{32.737}{15} = 2.1825$$

$$\bar{x}_g = \text{antilog}(2.1825) \cong 152,$$

median = 130,

$$\bar{x} = \frac{\Sigma x_i}{n} = \frac{6632}{15} = 442.$$

MPN THEORY, TABLES, AND REPORTING

I DERIVATION OF THE MPN

A Assumptions

The validity of the MPN procedure is based upon two principal assumptions.

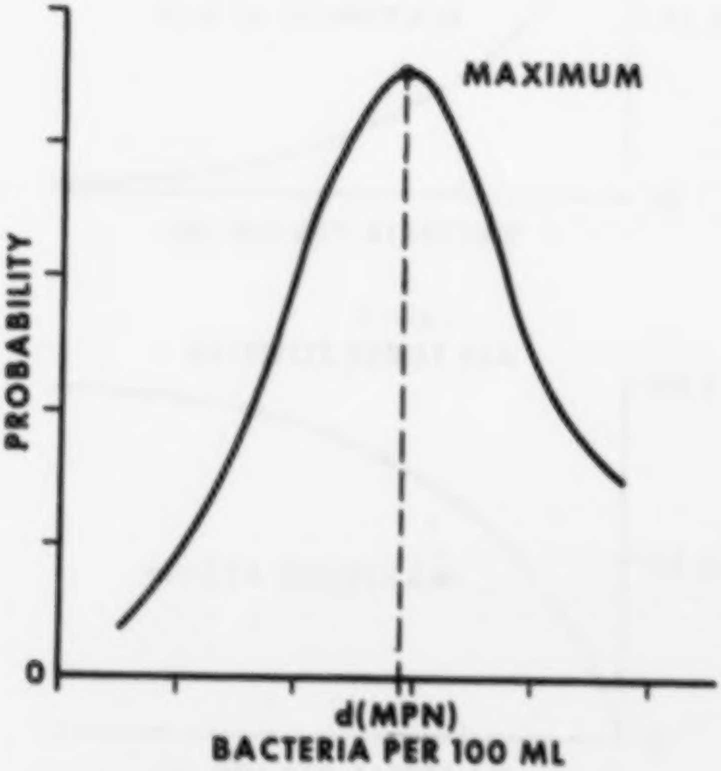
1 In statistical language, the first is that the organisms are distributed randomly throughout the liquid. This means that an organism is equally likely to be found in any part of the liquid, and that there is no tendency for pairs or groups of organisms either to cluster together or to repel one another.

2 The second assumption is that each sample from the liquid, when incubated in the culture medium, is certain to exhibit growth whenever the sample contains one or more organisms.

B The Probability Equation

Based upon these assumptions, an equation for the probability of the observed combination of positive and negative tubes can be derived as a function of the true density δ. By solving this equation for different values of δ a curve can be plotted as shown in Figure 1.

Figure 1



Curves of this type always have a single maximum or peak. The value of δ, say d, which corresponds to the peak of the curve is called the most probable number, commonly designated as MPN.

The MPN is "most probable" in the sense that it is the number which maximizes the probability of the observed results. It is interesting to note that although the original derivation of the MPN predates modern statistical estimation, the MPN procedure corresponds to the currently accepted estimation procedure known as the "method of maximum likelihood."

C Indeterminant solutions

The MPN provides a meaningful estimate of δ only if there are both positive and negative tubes in at least one dilution. If all tubes are negative, the maximum of the probability curve occurs when δ is set equal to zero (see Figure 2) and thus the MPN is zero. If all tubes are positive, the maximum of the probability curve occurs when δ is set equal to infinity (see Figure 3) and thus the MPN is infinity.

Figure 2

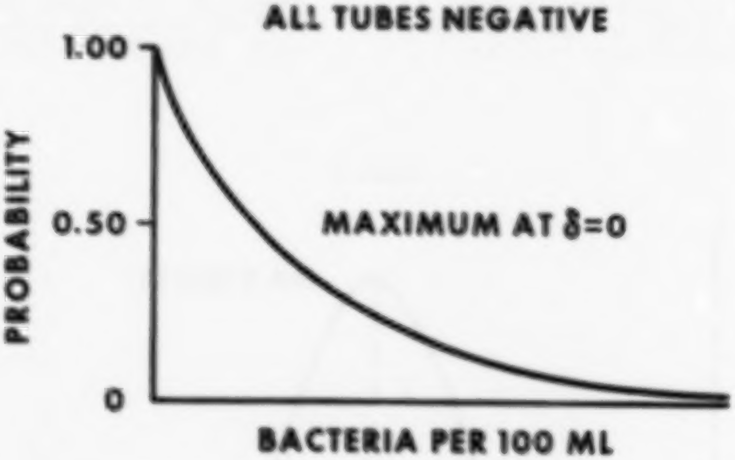
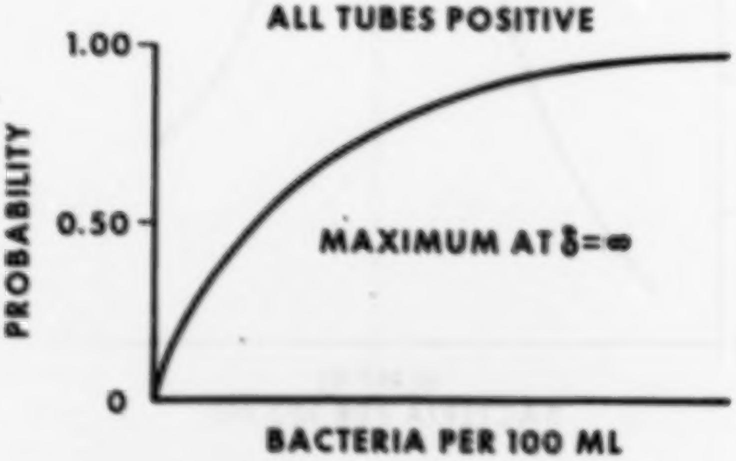


Figure 3

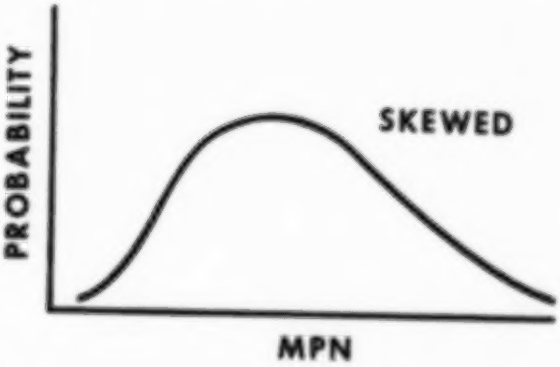


II DISTRIBUTION OF MPN VALUES

A Skewed Distribution

If a very large number of independent MPN determinations were made on the same water sample, the distribution of the MPN values would be such that very high values relative to the median value would occur more frequently than very low values. Thus the distribution of MPN values is skewed to the right as shown in Figure 4.

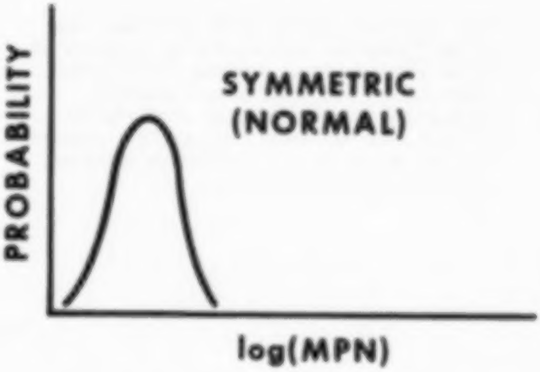
Figure 4



B Logarithmically Normal Distribution

Since it is mathematically inconvenient to work with data distributed asymmetrically, it is desirable to transform the skewed data in such a way that the transformed values have a symmetric distribution resembling the normal. In the case of MPN values the logarithms of the MPN's are approximately normally distributed as shown in Figure 5.

Figure 5



C Precision of MPN Estimates

The lack of precision of MPN estimates of bacterial densities is generally recognized. A measure of the precision is given by the confidence limits on the estimate which can be computed on the basis of the normal distribution of the logarithms of the MPN values. It has been verified that three-tube and five-tube MPN estimates are approximately logarithmically normal and the standard deviation of the logarithms of the MPN's is given by the formula:

$$\sigma_{\log} = \frac{0.58}{\sqrt{n}} \,.$$

where $\sigma_{\log}$ is the standard deviation of the logarithms of the MPN estimates and n is the number of tubes in each dilution.

The upper and lower 95% confidence limits of an MPN estimate are given by the formulas:

$$\begin{aligned} \text{UCL} &= \text{antilog}\,(\log \text{MPN} + 1.96\,\sigma_{\log}) \\ &= \text{MPN} \cdot k, \\ \text{LCL} &= \text{antilog}\,(\log \text{MPN} - 1.96\,\sigma_{\log}) \\ &= \text{MPN} \div k, \end{aligned}$$

where $k = \text{antilog}\,(1.96\,\sigma_{\log})$.

Notice that the confidence limits are not symmetric about the MPN estimate.

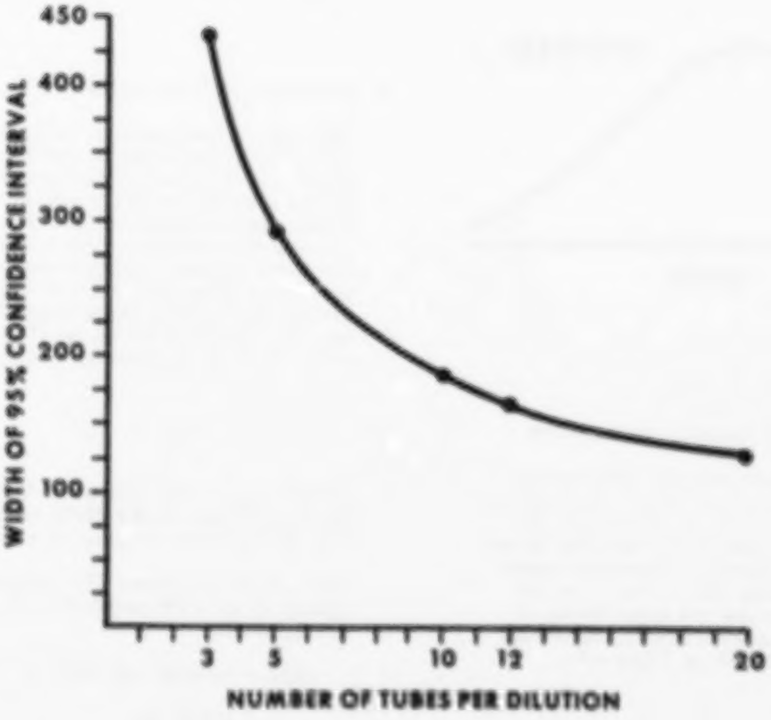
The precision of the MPN estimate can be increased by increasing the number of tubes per dilution. Figure 6 shows the width of the 95% confidence interval expressed as a percentage of the MPN estimate for various values of n. Notice that the width of the confidence interval decreases as n increases.

III PLANNING A DILUTION SERIES

A The Rationale

It was mentioned that the MPN procedure provides a reasonable estimate of the true density only if there are both positive and negative tubes in at least one dilution. It follows that in a series of dilutions the expected number of organisms in the highest sample volume (lowest dilution) should be at least one, otherwise all tubes may be negative and the result will be an indeterminant value.

Figure 6



Similarly, the expected number or organisms in the lowest sample volume (highest dilution) ν_L should not exceed one, to avoid the risk that all tubes will be positive.

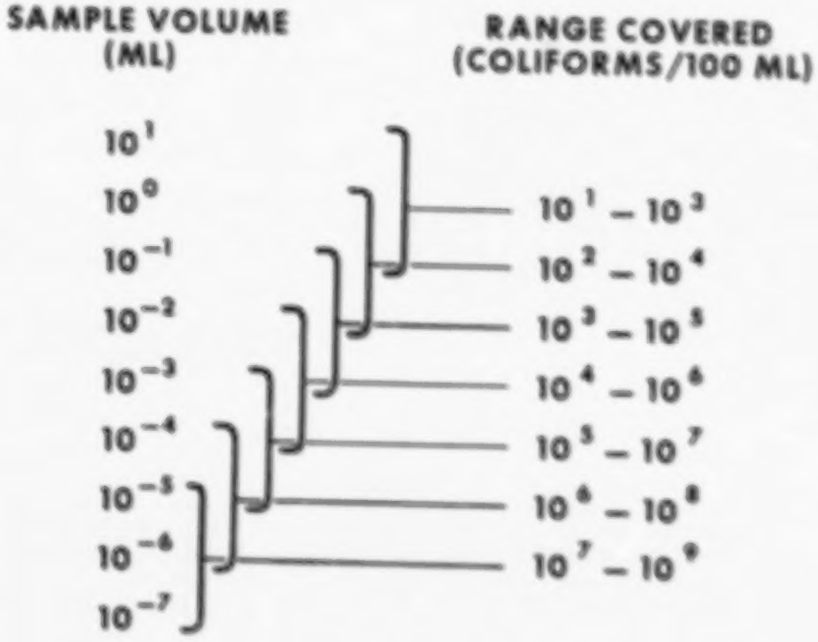
B The Rule

The above line of reasoning leads to the rule that a dilution series is capable of estimating any density between $1/\nu_H$ and $1/\nu_L$. In practice, we use the rule by first guessing two limits δ_H and δ_L between which we are fairly certain that the actual density lies. The sample volumes are then chooseen to satisfy the rules

$$\nu_H \geq \frac{1}{\delta_L} \; ; \; \nu_L \leq \frac{1}{\delta_H} .$$

Table 1 displays the range of densities covered by various decimal dilution series.

Table 1


SAMPLE VOLUME (ML)
RANGE COVERED (COLIFORMS/100 ML)
10^{1}
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
$10^{1} - 10^{3}$
$10^{2} - 10^{4}$
$10^{3} - 10^{5}$
$10^{4} - 10^{6}$
$10^{5} - 10^{7}$
$10^{6} - 10^{8}$
$10^{7} - 10^{9}$

IV MPN TABLES

Because the computations involved in estimating bacterial densities from fermentation tube results are so repititious and time-consuming, it is common laboratory practice to use Tables of Most Probable Numbers. Most MPN tables are based on 3 sample volumes in decreasing decimal increments. Though tables can be prepared for any desired series of sample increments, the sample volumes most commonly considered are 10 ml, 1.0 ml, and 0.1 ml.

In addition, tables can be devised for different numbers of replicate inoculations of each sample volume. For example, the MPN table most commonly used is based on five replicate 10 ml portions, five 1.0 ml portions, and five 0.1 ml portions. A separate table is required for another combination of sample volumes, consisting of five replicate 10 ml portions, one 1.0 ml portion, and one 0.1 ml portion. This is often used in bacteriological potability tests on water.

The MPN tables in the 12th edition of Standard Methods for the Examination of Water and Wastewater consist of the MPN indices and 95% confidence limits for various combination of positive and negative tubes. In the remainder of this outline we will consider only the table for five 10 ml portions, five 1.0 ml portions, and five 0.1 ml portions (see Table 5). However, the principles presented apply equally well to the other tables in Standard Methods.

V USE OF TABLES OF MOST PROBABLE NUMBERS

If five 10 ml portions, five 1.0 ml portions, and five 0.1 ml portions are inoculated initially, and positive results are secured from five of the 10 ml portions, three of the 1.0 ml portions, and none of the 0.1 ml portions, then the coded result of the test is 5-3-0. The code can be looked up in the MPN table and the MPN per 100 ml recorded directly. When more than three dilutions are employed in a decimal series of dilutions, only three of these are significant. To select the three dilutions to be employed in determining the MPN index, the following rules apply:

A The highest dilution (smallest sample volume) which gives positive results in all of the five portions tested - no lower dilution giving any negative results - and the two next succeeding higher dilutions should be choosen.

B If the lowest dilution (largest sample volume) tested does not give positive results in all of the five portions tested, then it and the two next succeeding higher dilutions should be choosen.

C If a positive result occurs in a dilution higher than the three choosen according to rules (1) or (2), it should be included in the result of the highest choosen dilution.

The examples presented in Table 2 illustrate the application of these rules.

Table 2

No. ml sample per tube → No. tubes per sample vol →	100 5	10 5	1.0 5	0.1 5	0.01 5	0.001 5	Code	See Below
No. tubes in sample giving positive results in test		5	4	1			5-4-1	
	5	5	4	0	0	0	5-4-0	(1)
		4	1	0	0	0	4-1-0	(2)
	5	5	4	1	1	0	5-4-2	(3)
		5	5	5	4		5-5-4	(4)
		5	5	5	5		5-5-5	(5)
		0	0	0	0		0-0-0	(6)
		0	1	0	0		0-1-0	(7)
		1	0	0	0		1-0-0	(8)

Discussion of examples:

1 When all the inoculated tubes of more than one of the decimal series give positive results, then it is customary to select the smallest sample volume (here, 10 ml) in which all tubes gave positive results. The results of this volume and the next lesser volumes are used to determine the coded result.

2 When none of the sample volumes gives positive results in all increments of the series, then the results obtained are used to designate the code. Note that it is not permissible to assume that if the next larger increment had been inoculated, all tubes probably would have given positive results and therefore assign a 5-4-1 code to the results.

3 Here the results are spread through four of the sample volumes. In such cases, the number of positive tubes in the smallest sample volume is added to the number of tubes in the third sample volume (counting down from the smallest sample volume in which all tubes gave positive results).

4 Here it is necessary to use the 5-5-4 code, because inoculations were not made of 0.001 ml sample volumes; and it is not permissible to assume that if such sample volumes had been inoculated, they would have given negative results, or any other arbitrarily-designated result.

5 This is an indeterminate result. Many MPN tables do not give a value for such a result. If the table used does not have the code, then look up the result for code 5-5-4, and report the result "greather than" the value shown for the 5-5-4 code.

6 Like (5), this is an indeterminate result. If the code does not appear in the table being used, then look up the result for code 1-0-0, and report the MPN as "less than" the value shown for the 1-0-0 code.

7 The current edition of Standard Methods stipulates this type of code designation when unusual results such as this occur.

8 Note the difference from (7) above. Inoculations of 100 ml portions were not made, and it cannot be assumed that the result would have called for code 0-1-0.

To determine the MPN from the coded result the following rules apply:

a If the first number in the coded result represents a 10 ml sample volume, then the MPN per 100 ml is read directly from the table.

b If the coded result represents a series of decimal dilutions other than 10 ml, 1.0 ml, and 0.1 ml, record the value given by the formula:

$$\text{MPN (from table)} \times \frac{10}{\text{largest vol. used}} = \text{MPN per 100 ml.}$$

VI OCCURRENCE OF IMPROBABLE TUBE RESULTS

Many of the theoretically possible tube results are omitted from MPN tables. For example, codes 0-0-3, 0-0-4, 0-0-5, and 5-0-5 are not included as well as many others. These are omitted because the theoretical probability of such results is so low as to exclude them from practical consideration.

The frequency of occurrence of various code results is shown in Table 3 both on a theoretical basis and on the basis of actual laboratory experience. Table 3 suggests that the codes omitted from most MPN tables can be expected to occur about one percent of the time. If, in reviewing laboratory data, the theoretically unlikely codes occur appreciably more than one percent of the time, there is an indication for inquiry into the causes. Table 4 indicates maximum permissible numbers of unlikely codes for various numbers of samples tested.

Table 3

FIVE-TUBE AND THREE-TUBE CODES THAT INCLUDE 99 PER CENT OF ALL RESULTS

Group	Theoretically Expected Percentage of Results	Theoretically Expected Cumulative Percentage	Observed Percentage of 360 Samples
	Five-Tube-Test		
Class 1 codes 550, 551, 552, 553, 554, 500, 510, 520, 530, 540, 100, 200, 300, 400.	67.5	67.5	68.0
Class 2 codes 511, 521, 531, 541, 542, 110, 210, 310, 410, 420.	23.6	91.1	23.1
Class 3 codes 501, 010, 532, 320, 522, 220, 543, 430, 120, 533, 330, 502, 020, 544, 440, 301, 401, 431, 201, 411, 101, 311, 421, 211, 001.	7.9	99.0	7.5
Improbable codes	1.0	100.0	1.4
	Three-Tube Test		
Class 1 codes 330, 331, 332, 300, 310, 320, 100, 200.	81.5	81.5	81.7
Class 2 codes 321, 311, 301, 210, 110, 010.	14.9	96.4	14.1
Class 3 codes 322, 220, 201, 101 312, 120.	2.7	99.1	3.7
Improbable codes	0.9	100.0	0.6

Table 4

Number of Samples	Maximum Number of Improbable Codes
1 - 15	1
16 - 45	2
46 - 83	3
84- 130	4
131- 180	5
181- 233	6
234- 290	7
291- 350	8
351- 413	9
414- 477	10
478- 543	11

Table 5

MPN INDEX AND 95 PER CENT CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN FIVE 10-ML PORTIONS, FIVE 1-ML PORTIONS AND FIVE 0.1-ML PORTIONS ARE USED

Number of Tubes Giving Positive Reaction Out of			MPN Index per 100 Ml	95% Confidence Limits		Number of Tubes Giving Positive Reaction Out of			MPN Index per 100 Ml	95% Confidence Limits	
of 10 l Each	5 of 1 Ml Each	5 of 0.1 Ml Each		Lower	Upper	5 of 10 Ml Each	5 of 1 Ml Each	5 of 0.1 Ml Each		Lower	Upper
0	0	1	2	<0.5	7	4	2	1	26	9	78
0	1	0	2	<0.5	7	4	3	0	27	9	80
0	2	0	4	<0.5	11	4	3	1	33	11	93
1	0	0	2	<0.5	7	4	4	0	34	12	93
1	0	1	4	<0.5	11	5	0	0	23	7	70
1	1	0	4	<0.5	11	5	0	1	31	11	89
1	1	1	6	<0.5	15	5	0	2	43	15	114
1	2	0	6	<0.5	15	5	1	0	33	11	93
2	0	0	5	<0.5	13	5	1	1	46	16	120
2	0	1	7	1	17	5	1	2	63	21	150
2	1	0	7	1	17	5	2	0	49	17	130
2	1	1	9	2	21	5	2	1	70	23	170
2	2	0	9	2	21	5	2	2	94	28	220
2	3	0	12	3	28	5	3	0	79	25	190
3	0	0	8	1	19	5	3	1	109	31	250
3	0	1	11	2	25	5	3	2	141	37	340
3	1	0	11	2	25	5	3	3	175	44	500
3	1	1	14	4	34	5	4	0	130	35	300
3	2	0	14	4	34	5	4	1	172	43	490
3	2	1	17	5	46	5	4	2	221	57	700
3	3	0	17	5	46	5	4	3	278	90	850
4	0	0	13	3	31	5	4	4	345	120	1,000
4	0	1	17	5	46	5	5	0	240	68	750
4	1	0	17	5	46	5	5	1	348	130	1,000
4	1	1	21	7	63	5	5	2	542	180	1,400
4	1	2	26	9	78	5	5	3	918	300	3,200
4	2	0	22	7	67	5	5	4	1,600	640	5,800

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RESPONSIBILITIES OF A SURVEY OFFICER

Dale Van Donsel*

I OBJECTIVES OF WATER LABORATORY SURVEY PROGRAM

A Assure Laboratory Compliance With Public Health Service Drinking Water Standards (1962)

Potable water used by carriers is subject to the Interstate Quarantine Regulations.

B Public Health Aspects

Improve the quality of laboratory procedures for the greatest possible protection to the health of the consumer.

C Assistance To The States

1 Technical advice and assistance

2 Point out deviations from approved methodology to protect State from criticism of data reliability and laboratory prestige.

II THE MANY ROLES OF A LABORATORY SURVEY OFFICER

A Official Representative of the State

1 Assure compliance with "Standard Methods" and Drinking Water Standards.

2 Familiarity with laws and problems of local jurisdictions.

B Technical Consultant

1 Demonstrate technical procedures as needed

2 Familiarity with merits of new equipment and procedures

3 Source of technical literature and information

4 Aid in planning new local laboratory facilities.

C Economic Advisor

1 Advice best use of limited funds and resources

2 Help justify expenditures to local jurisdictions.

D Educator

1 Informal instructions during laboratory surveys

2 Formal training sessions and training in State Health Department Laboratories.

E Public Relations Man

1 Local laboratories should feel free to request help when problems arise.

2 Engineering staff should be aware of importance of competent laboratory service.

3 State officials should be aware of public health implications of local laboratories to the health of the State.

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Water Supply Research Section, NCUI

III SPECIFIC PROBLEM AREAS IN WATER LABORATORIES

A Most Frequent Deviations from "Standard Methods" in Laboratories Using the Multiple Tube Procedure

Items	% Deviation	
	60 State and Regional	70 Municipal
Sample bottles (size, air space)	56.7	30.1
Distilled water suitability	51.7	25.7
Application of completed MPN	46.7	41.4
Sample transit time	45.0	8.6
Confirmed test procedure	36.7	41.4
Buffered dilution water	31.7	31.4
Incubator (temp., record, maintenance)	28.3	65.7
Clean glassware	18.3	25.7
Media pH (pH equipment, pH record)	15.0	70.0
Sample dechlorination	11.7	42.9
Autoclave (procedure, maintenance)	11.7	50.0
Lactose broth preparation	10.0	24.3
Media sterilization	8.3	20.0
Brillant green lactose preparation	6.7	22.9
Media storage	1.7	28.6
Sampling frequency	1.7	12.5

B Most Frequent Deviations in Laboratories Using the Membrane Procedure

1 Excessive sterilization of membrane

2 Estimation of sample volumes

3 Funnel rinsing techniques

4 Insufficient humidity during incubation when using glass dishes

5 Improper light source for counting membranes.

C Cumulative Effect of a Number of Small Deviations on Recovery of Attenuated Coliforms Can Cause Failure to Detect a Contaminated Water Supply.

IV SOME RECOMMENDATIONS TO BE INCLUDED IN FUTURE LABORATORY EVALUATIONS

A Dechlorination of Samples

A 0.1% iodine solution to check for sodium thiosulfate in sample bottles.

B Transportation and Storage

1 Icing of samples whenever possible

Time and temperature effects on coliforms in samples are variable.

2 Alternate Means of Transportation for Samples from areas beyond 1-day postal service can be arranged.

C Record of Laboratory Examinations

How to evaluate occurrence of unusual MPN combinations in a laboratory's records.

D Distilled Water Suitability Test

Some additional reasons for its application.

E Drying Membranes Before Counting

May be appealing to inexperienced personnel, but can result in false positives.

F Maintaining Proficiency in Examining Membrane Filters

An occasional polluted water sample in laboratories having few positive samples.

G Standard Plate Count

Is any use made of the data?

H Report to Laboratories

1 Prompt submission of report has the greatest impact on a laboratory.

2 Standard recommendations in "Evaluation of Water Laboratories" are a guide in writing narrative section of report, but local practices and problems may require more explanations and recommendations.

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WATER QUALITY BEHAVOIR IN RESERVOIRS

James M. Symons*

I CONTRAST STREAM BEHAVIOR TO RESERVOIR BEHAVIOR

A Depth

B Detention Time

C Thermal Stratification

II BENEFICIAL INFLUENCES OF RESERVOIRS

A Turbidity Reduction

B Hardness Reduction

C Organic Oxidation

  1 BOD Reduction

  2 Color Reduction

D Coliform Reduction

E Smoothing

III ADVERSE INFLUENCES OF RESERVOIRS

A Less Mixing

B Lower Reaeration

C Backup of Pollutants

D Algal Blooms

  1 Aesthetics

  2 Tastes and Odors

E No Bottom Scour

F Thermal Stratification

IV INFLUENCE OF THERMAL STRATIFICATION

A Low Dissolved Oxygen

  1 Iron and manganese dissolution

  2 Hydrogen Sulfide production

  3 Carbon Dioxide increase

  4 pH reduction

  5 Organic persistance

  6 Shortcircuiting

V CURRENT RESEARCH

A Survival of Indicator Organisms in Stratified Reservoir Waters

B Survival of Bacterial Pathogens in Bottom Muds

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*Chief, Surface Water Protection Activities, WSSR

ALGAE AND ACTINOMYCETES IN WATER SUPPLIES

I Water treatment always should include detection and control of microorganisms.

A Two types of microorganisms are involved:

1 Pathogenic type includes such forms as the typhoid bacteria, the dysentery ameba, and the infectious hepatitis virus.

2 Interference type includes taste and odor organisms, filter-clogging organisms, pipe-infesting organisms, and others.

B Water treatment practices are closely associated with these organisms.

1 For pathogens, practices include coliform tests, use of chlorine, and guarding the water supply against sewage pollution.

2 For interference organisms, practices include plankton enumeration, use of copper sulfate and the covering of reserviors.

3 Many of the other treatment practices have significant effects on the organisms.

C This discussion will be limited to the interference organisms.

II EXAMPLES OF PROBLEMS CAUSED BY INTERFERENCE ORGANISMS

A At Chicago, the alga Dinobryon reappears almost every year, generally in June and July in numbers sufficient to impart a prominent fishy odor to the water. In 1951, it required an estimated $70, 500 worth of activated carbon to control the odor of this organism for a period of two months.

B At Indianapolis, copepods were present in parts of the distribution system in numbers sufficient to be visible in the drinking water. The eggs of the copepods were found to pass through the filters and to hatch in the distribution system.

C At Oklahoma City, prominent earthy odors have appeared frequently. The organisms blamed for this trouble are the mold-like actinomycetes.

D At Peoria, white wigglers up to 3/8" long were reported in the tap water, during early March, 1956. These chironomid larvae had hatched in the city's open reservoir, requiring that the reservoir be drained, cleaned and treated with a larvicide.

E At Chicago, diatoms are a very important cause of short filter runs. The one diatom Tabellaria is considered to be more responsible than any other organism for this trouble.

F In Ontario, the alga Cladophora often grows in large numbers attached to rocks on the shoreline of lakes. When the alga is broken loose it collects near the shoreline and gives rise to very offensive odors.

G In a water supply impoundment in Utah the plankton algae frequently cause the pH of the water to increase to 8.3 or higher, requiring that the water be treated with acid to obtain the desired pH of 8 or lower.

H In Texas a water supply from underground sources was stored in a large open settling basin. Oscillatoria and unicellular green algae developed in large numbers in the stored water, turning it green and producing a strong odor.

I Los Angeles has more than 25 open reservoirs of various sizes and ranging in elevation from almost sea level to over

7,000 feet. Many tons of copper sulfate are used every year in these reservoirs for rigid control of plankton, chiefly diatoms and occassionally blue-green algae. This treatment is carried out to improve the water quality including the reduction of tastes and odors.

III TYPES OF PROBLEMS CAUSED BY INTERFERENCE ORGANISMS

A Tastes and Odors

1 May be caused by algae, actinomycetes, crustacea, and anaerobic bacteria.

2 Common algal odors imparted to water are ones described as fish, earthy, musty, grassy, cucumber, geranium, nasturtium, and septic.

3 Common actinomycete odor is earthy.

4 Tastes produced in water by algae include sweet and bitter.

5 Other causative agents of tastes and odors may be industrial wastes, sludge, and compounds dissolved from soil and rock, and chemicals used in treatment.

B Filter Clogging

1 Both rapid and slow sand filters are affected.

2 Diatoms are the organisms most frequently involved but blue-green algae, filamentous green algae and other organisms as well as silt may cause it.

C Other Problems in the Treatment Plant

1 Algae may cause variation in the pH, hardness, color, and organic content of the water.

2 Amount of plankton organisms often influences the rate and effectiveness of coagulation.

3 Chlorine dosage may depend upon amount of plankton organisms present.

4 Growths of algae may reduce the flow through influent channels and screens.

5 Organisms may be responsible for increasing the quantity of sludge to be disposed of in sedimentation basins.

D Infestation of Distribution Systems

1 Attached organisms reduce the rate of flow in the pipes.

2 Iron and sulfur bacteria may initiate or stimulate corrosion of pipes.

3 Organisms may appear as visible bodies in tap water.

4 Tastes and odors may result from presence of organisms.

5 Chlorine residual is difficult to maintain when organic matter is present.

6 Organisms could theoretically harbor and protect against chlorine certain pathogenic bacteria.

E Profuse Growths of Organisms in Raw Water Supplies

1 A limited and balanced growth of various organisms is generally an asset.

2 Extensive surface mats, blooms and marginal growths often cause troubles along the shoreline and eventually in the treatment plant.

3 Some fish kills may be caused by profuse growths of algae by reducing the DO during the night.

4 Certain massive growths of blue-green algae are deadly poisonous to animals.

IV ORGANISMS INVOLVED

A Animal forms include protozoa, rotifers, crustaceans, worms, bryozoans, fresh water sponges, water mites and larval stages of various insects.

B Plant forms include algae, actinomycetes and other bacteria, molds and larger aquatic green plants.

V IMPORTANCE OF BIOLOGICAL PROBLEMS

A The increased use of surface water supplies increases the problems caused by organisms. Biological problems are less common with ground water supplies.

B Standards of water quality requested by domestic and industrial patrons are rising.

C Procedures for detection, control and prevention of problems caused by organisms are improving and are receiving more extensive use.

VI A number of methods may be used to control the interference organisms or their products:

A Addition to water or an algicide or pesticide such as copper sulfate, chlorine dioxide or copper-chlorine-ammonia.

B Mechanical cleaning of distribution lines, settling basins, sand filters, screens, and reservoir walls.

C Modification of coagulation, filtration, chemical treatment, or location of intake.

D Use of absorbent, such as activated carbon, for taste and odor substances.

E Modification of Reservoir to Reduce the Opportunities for Massive Growths of Algae

1 By covering treated water reservoir to exclude sunlight

2 By increasing the depth of the water in reservoirs

3 By eliminating shallow marginal areas

4 By reducing the amount of fertilizing nutrients entering the reservoir.

VII It is generally more satisfactory to anticipate and prevent problems due to these organisms than it is to cope with them later.

A Routine biological tests are essential to detect the initial development or presence of interference organisms.

1 Control measures can then be used before problem becomes acute.

2 These tests should be applied to the raw treatment plant water supply and distribution system.

B In the Reservoir or Other Raw Water Supply

1 Routine plankton counts should be made of water samples from selected locations. Plankton counter should be aware of the particular organisms known to be most troublesome.

2 During the warmer months routine surveys of the reservoir, lake or stream should be made to record any visible growths of algae and other organisms.

3 Odor tests of water from several locations should be made to obtain advance notice of potential trouble at the treatment plant.

C In the Treatment Plant

1 Records of plankton counts and threshold odor between each step in treatment gives data on effectiveness of each procedure.

2 Coagulation and filtration can be adjusted to remove up to 95% or more of organisms in water.

3 Microscopic analysis of samples of filter material for organisms can supply data useful in modifying sand filtration and treatment of finished water.

D In the Distribution System With Its Finished Water

1 Open reservoirs require constant attention especially during summer.

2 Parts of the system farthest from the treatment plant or adjacent to dead ends require most frequent sampling for organisms and tastes and odors.

VIII SUMMARY

A Interference organisms cause problems in distribution systems, treatment plants, raw water supplies.

B Organisms involved include algae, actinomycetes, other bacteria, and minute aquatic animals.

C Control is by special chemicals, mechanical cleaning, adjustment of chemical or mechanical treatment and by modification of reservoirs, intakes, etc., for the raw water supply.

D Facilities for detection of problems in their early stages are required for most efficient and satisfactory control.

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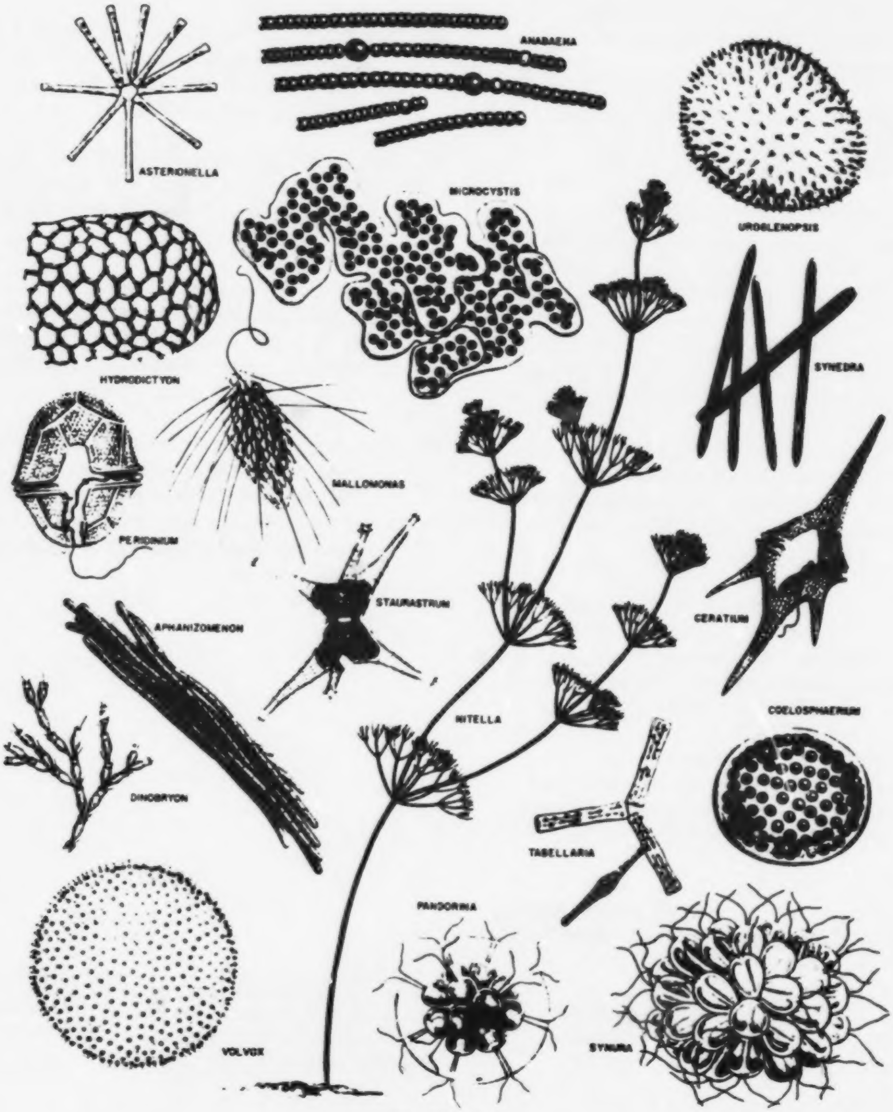
This outline was prepared by C.M. Palmer, Aquatic Biologist, Biological Treatment Research Activities, Cincinnati Water Research Lab.

ALGAE IMPORTANT IN WATER SUPPLIES

TASTE AND ODOR ALGAE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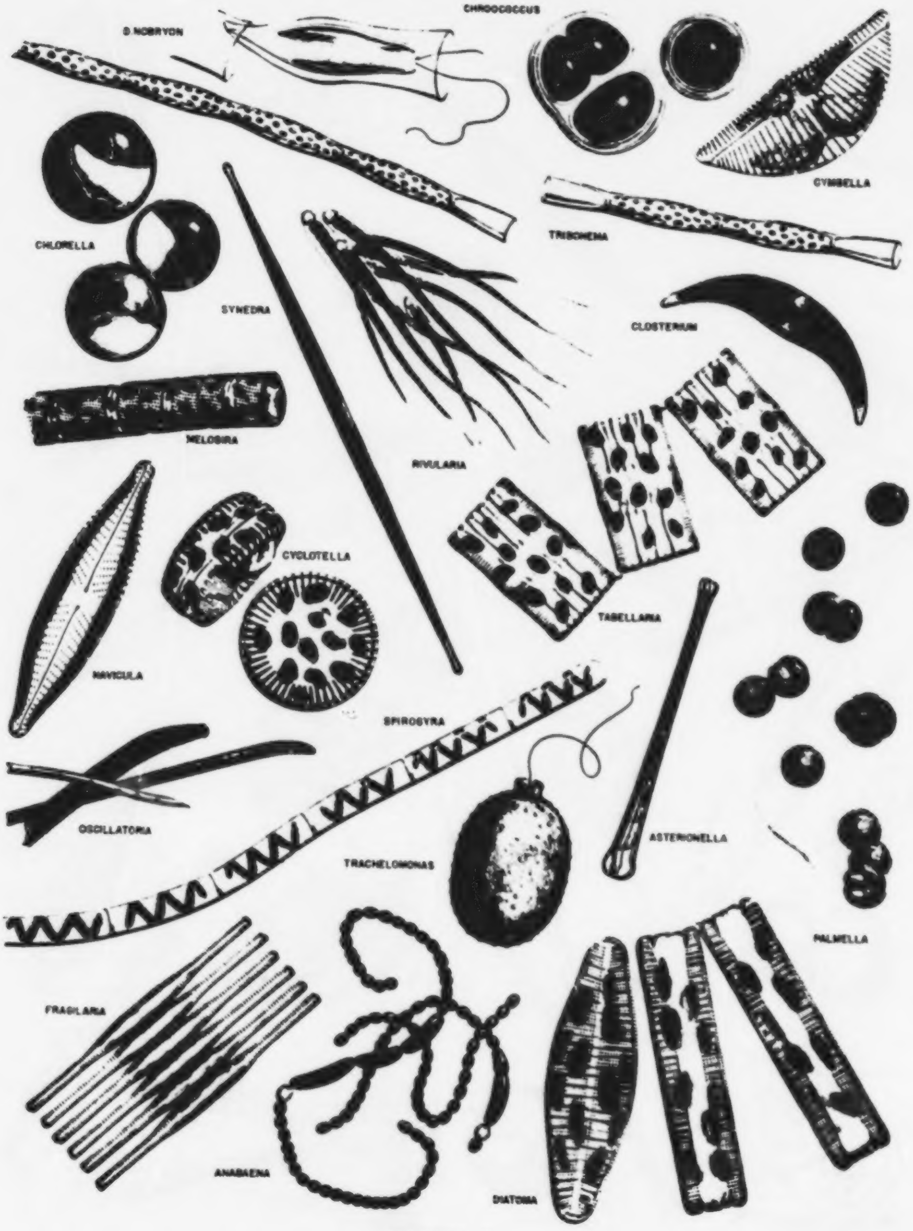
STAURASTRUM
APHANIZOMENON
CERATIUM
NITELLA
COELOSPHAERIUM
DINOBRYON
TABELLARIA
PANDORINA
VOLVOX
SYNURA

PLATE I

FILTER CLOGGING ALGAE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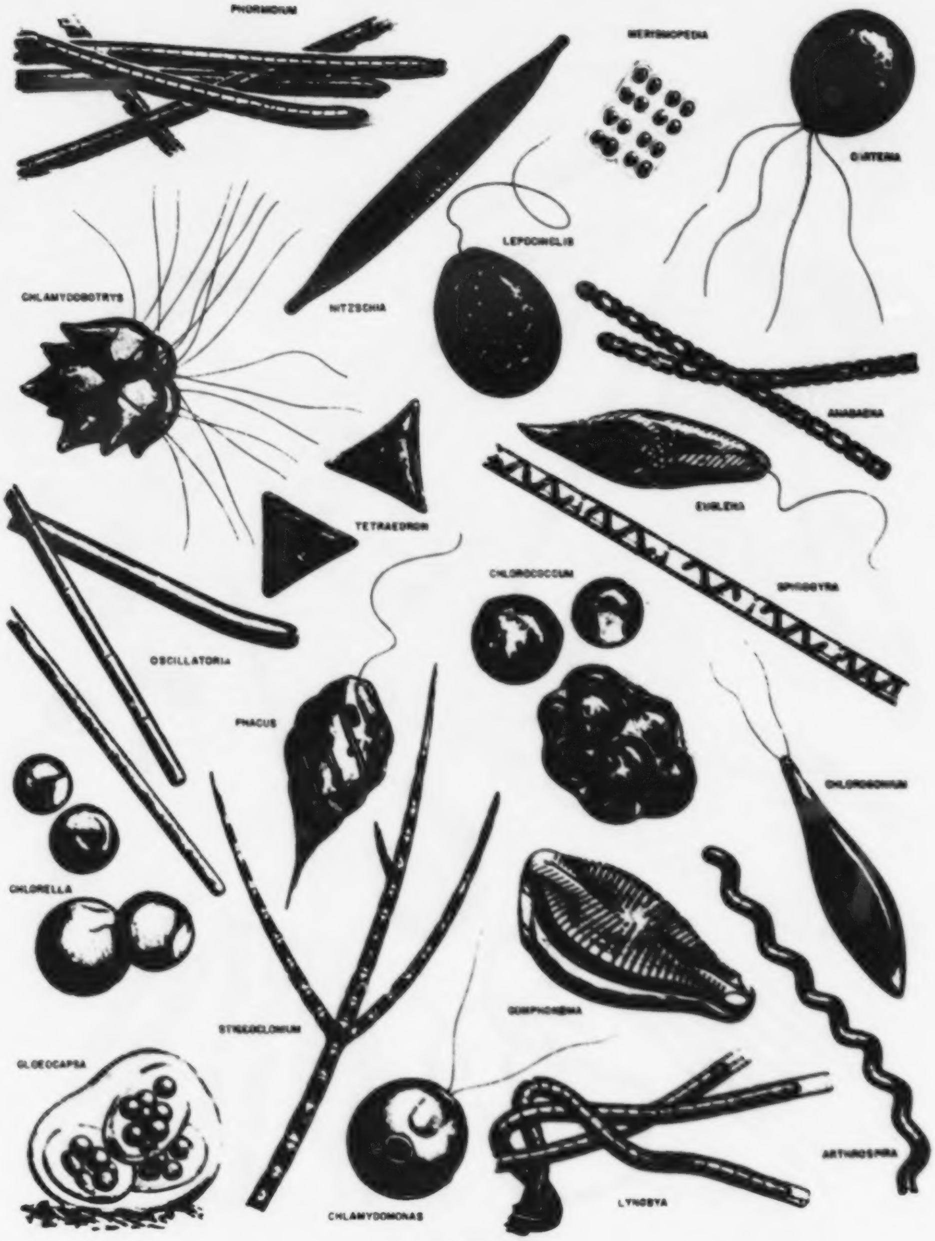
CYCLOTELLA
TABELLARIA
NAVICULA
SPIROGYRA
OSCILLATORIA
TRACHELOMONAS
ASTERIONELLA
PALMELLA
FRAGILARIA
ANABAENA
DIATOMA

PLATE 2

POLLUTED WATER ALGAE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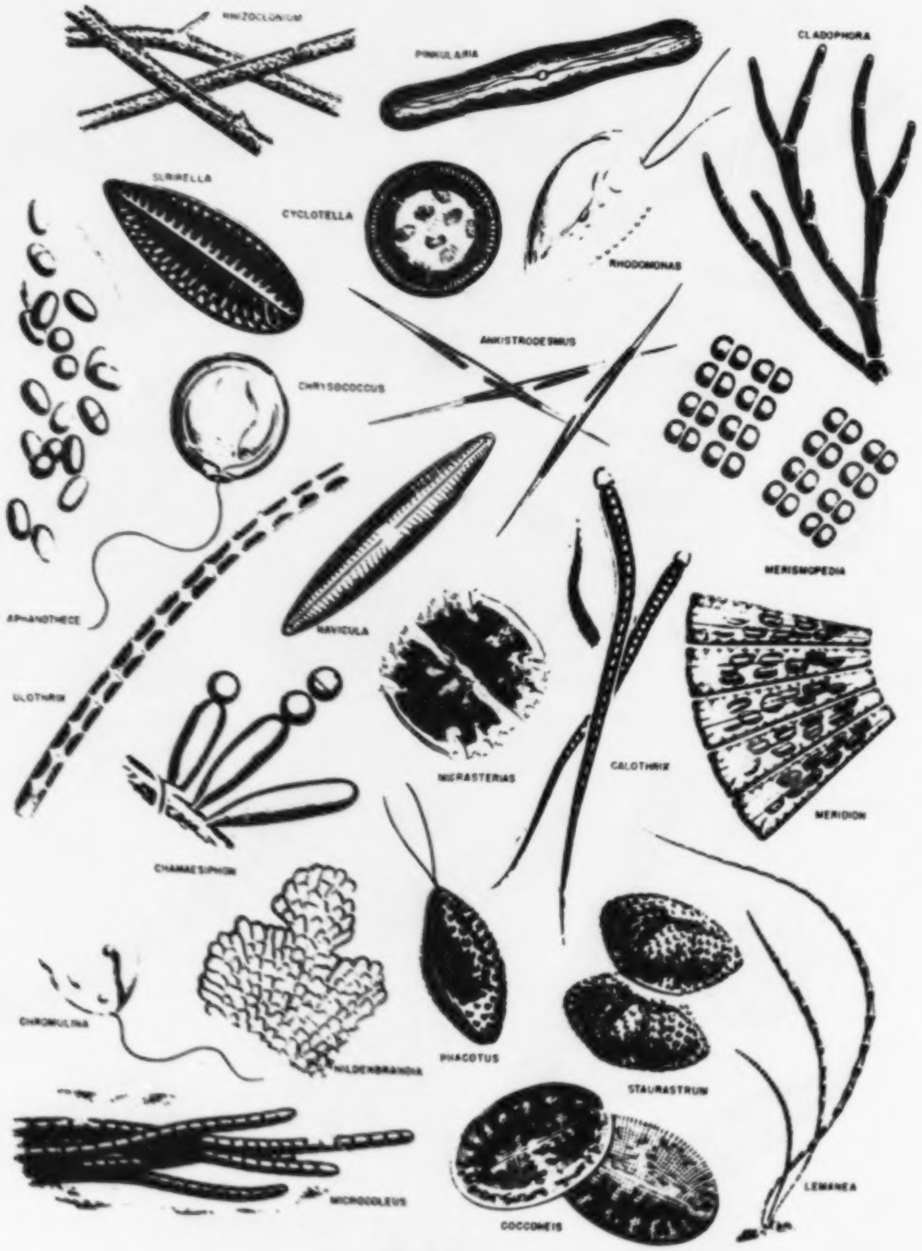
SPIROGYRA
CHLOROCOCCUM
OSCILLATORIA
PHACUS
CHLOROGONIUM
CHLORELLA
GOMPHONEMA
STIGEOCLONIUM
GLOEOCAPSA
CHLAMYDOMONAS
LYNGBYA
ARTHROSPIRA

PLATE 3

CLEAN WATER ALGAE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CYCLOTELLA
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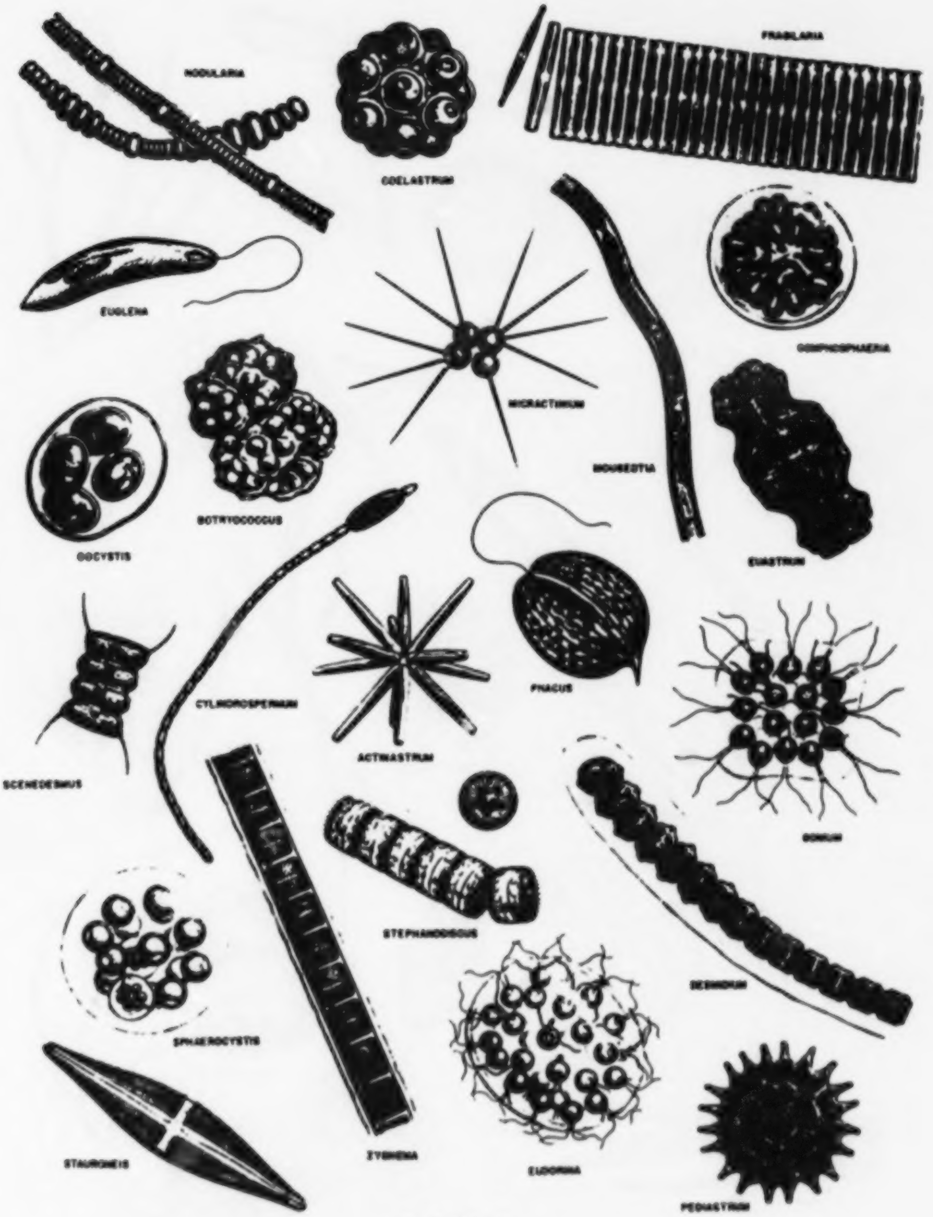
CHRYSOCOCCUS
MERISMOPEDIA
APHANOTHECE
NAVICULA
ULOTHRIX
MICRASTERIAS
CALOTHRIX
MERIDION
CHAMAESIPHON
CHROMULINA
HILDENBRANDIA
PHACOTUS
STAURASTRUM
LEMANEA
MICROCOLEUS
COCCONEIS

PLATE 4

SURFACE WATER ALGAE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MOUGEOTIA
GOMPHOSPHAERIA
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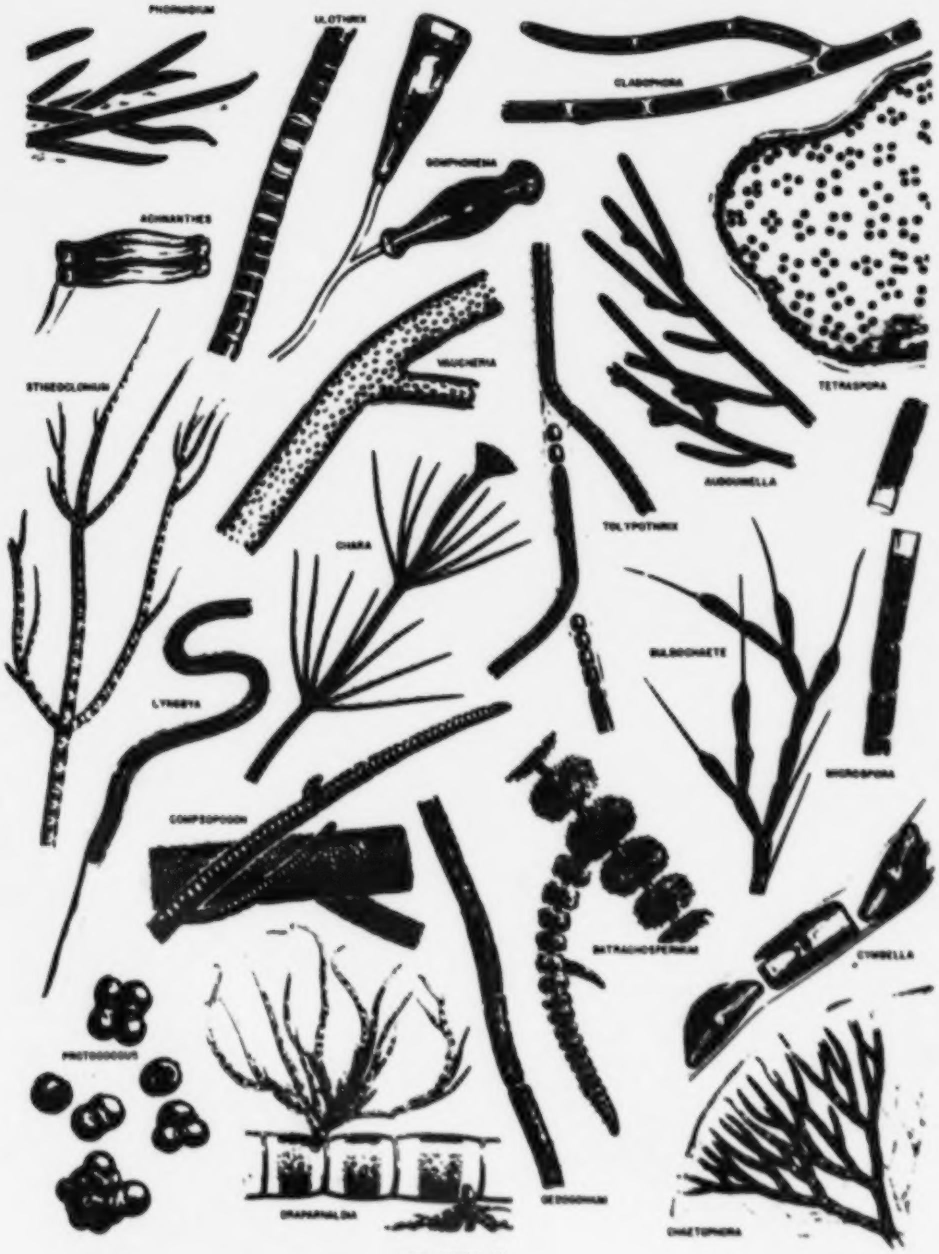
EUASTRUM
SCENEDESMUS
CYLINDROSPERMUM
ACTINASTRUM
PHACUS
SYNURA
STEPHANODISCUS
DESMIDIUM
SPHAEROCYSTIS
STAURONEIS
ZYGNEMA
EUDORINA
PEDIASTRUM

PLATE 5

ALGAE GROWING ON RESERVOIR WALLS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STIGEOCLONIUM
VAUCHERIA
TETRASPORA
AUDOUINELLA
TOLYPOTHRIX
CHARA
BULBOCHAETE
LYNGBYA
MICROSPORA
COMPSOPOGON
BATRACHOSPERMUM
CYMBELLA
PROTOCOCCUS
DRAPARNALDIA
OEDOGONIUM
CHAETOPHORA

PLATE 6

ANIMAL PLANKTON

I INTRODUCTION

A Planktonic animals or zooplankton are found in nearly every major group of animals.

1 Truly planktonic species (euplankton) spend all or most of their active life cycle suspended in the water. Three groups are predominantly involved in fresh water; the protozoa, rotifers, and microcrustacea.

2 Transient planktonic phases such as floating eggs and cysts, and larval stages occur in many other groups.

B Many forms are strictly seasonal in occurrence.

C Certain rare forms occur in great numbers at unpredictable intervals.

D Techniques of collection, preservation, and identification strongly influence the species reported.

E In oceanographic work, the zooplankton is considered to include many relatively large animals such as siphonophores, ctenophores, hepteropods, pteropods, arrowworms, and euphausid shrimp.

F The plant-like or phytoplankton on the other hand are essentially similar in all waters, and are the nutritional foundation for the animal community.

II PHYLUM PROTOZOA

A The three typically free living classes, Mastigophora, Rhizopoda, and Ciliophora, all have planktonic representatives. As a group however, the majority of the phylum is benthic or bottom-loving. Nearly any of the benthic forms may occasionally be washed up into the overlying waters and thus be collected along with the euplankton.

B Class mastigophora, the non-pigmented zooflagellates

These have frequently been confused with the phytomastigina or plant-like flagellates. The distinction is made here on the basis of the presence or absence of chlorophyll as suggested by Palmer and Ingram 1955. (Note Figure: Non-Pigmented, Non-Oxygen Producing Protozoan Flagellates in outline No. 23: Oxygen Relationships of Plankton).

1 Commonly encountered genera

Bodo

Peranema

2 Frequently associated with eutrophic conditions

C Class Rhizopoda - amoeboid protozoans

1 Forms commonly encountered as plankton:

Chaos *(Amoeba)*

Arcella *Centropyxis*

Difflugia Heliozoa

Euglypha

2 Cysts of some types may be encountered in water plants or distribution systems; rarely in plankton of open lakes or reservoirs.

D. Class Ciliophora

1 Certain "attached" forms often found floating freely with plankton:

Vorticella
Carchesium

2 Naked, unattached ciliates. *Halteria* one of commonest in this group. Various heavily ciliated forms (holotrichs) may occur from time to time such as *Colpidium*, *Enchelys*, etc.

3 Ciliates protected by a shell or test (testaceous) are most often recorded from preserved samples. Particularly common in the experience of the National Water Quality Sampling Network are:

Codonella fluviatile

Codonella cratera

Tintinnidium (usually with organic matter)

Tintinopsis

III PHYLUM ROTIFERA

A Some forms such as *Anuraea cochlearis* and *Asplanchna pridonta* tend to be present at all times of the year. Others such as *Notholca striata*, *N. longispina* and *Polyarthra platyptera* are reported to be essentially winter forms.

B Species in approximate order of descending frequency currently recorded by National Water Quality Sampling Network are:

Keratella cochlearis

Polyarthra vulgaris

Synchaeta pectinata

Brachionus quadridentata

Trichocerca longiseta

Rotaria sp.

Filinia longiseta'

Kellicottia longispina

Pompholyx sp.

C Benthic species almost without number may be collected with the plankton from time to time.

IV PHYLUM ARTHROPODA

A Class Crustacea

1 The Class Crustacea includes the larger common freshwater euplankton. They are also the greatest planktonic consumers of basic nutrients in the form of phytoplankton, and are themselves the greatest planktonic contribution to the food of fishes. Most of them are herbivorous. Two groups, the cladocera and the copepods are most conspicuous.

2 Cladocera (Subclass Branchiopoda, Order Cladocera) or Water Fleas

a Life History

(1) During most of the year, eggs which will develop without fertilization (parthenogenetic) are deposited by the female in a dorsal brood chamber. Here they hatch into minature adults which escape and swim away.

(2) As unfavorable conditions develop, males appear, and thick-walled sexual eggs are enclosed in egg cases called ephippia which can often endure freezing and drying.

(3) Sexual reproduction may occur at different seasons in different species.

(4) Individuals of a great range of sizes, and even ephippia, are thus en-

countered in the plankton, but there is no "larval" form.

b Seasonal variation - Considerable variation may occur between winter and summer forms of the same species in some cases. Similar variation also occurs between arctic and tropical situations.

c Forms commonly encountered as open water plankton include:

Bosmina longirostris and others

Daphnia galeata and others

Other less common genera are:

Diaphanosoma, Chydorus, Sida, Acroperus, Ceriodaphnia, Polyphemus, Bythotrephes, and the carnivorous Leptodora.

d Heavy blooms of Cladocerans may build up in eutrophic waters.

3 The copepods (order Copepoda) are the perennial microcrustacea of open waters, both fresh and marine. They are the most ubiquitous of animal plankton.

a Cyclops spp is the genus most often found by the National Water Quality Sampling Network activities. Eucyclops, Paracyclops, Diaptomus, Canthocamptus, Epischura, and Limnocalanus are other forms reported to be planktonic.

b Copepods hatch into a minute charhcteristic larvae called a nauplius which differs considerably from the adults. After five or six moults, the copepodid stage is reached, and after six more moults, the adult. These larval stages are often encountered and are difficult to identify.

B Class Insecta

1 Only a single species of insect can be ranked as a true plankton, this is the midge fly Chaborus (approx. 8 spp, formerly Corethra).

2 The larva of this insect has hydrostatic organs that enable it to remain permanently suspended in the water.

3 It is usually found in the depths during the daytime, but comes to the surface at night.

V OCCASIONAL PLANKTERS

A While the protozoa, rotifers, and microcrustacea make up the bulk of the plankton, there are many other groups as mentioned above that may also occur. Locally or periodically these may be of major importance. Examples are given below.

B Phylum Coelenterata

1 Polyps of the genus Hydra may become detached and float about hanging from the surface film or floating detritus.

2 The freshwater medusa Craspedacusta occasionally appears in lakes or reservoirs in great numbers.

C Phylum Platyhelminthes

1 Minute Turbellaria (relatives of the well known Planaria) are sometimes taken with the plankton

Reproduced from best available copy.

in eutrophic conditions. They are readily confused with ciliate protozoa.

2 Cercaria larvae of Trematodes (flukes) parasitic on certain wild animals, frequently appear in great numbers. When trapped in the droplets of water on a swimmer's skin, they attempt to bore in. Man not being their natural host, they fail. The resultant irritation is called "swimmer's itch". Some can be identified, but many unidentifiable species may be found.

3 In many areas of the world, cercaria larvae of human parasites such as the blood fluke *Schistosoma japonicum* may live as plankton, and penetrate the human skin directly on contract.

D Phylum Nemathelminthes

1 Nematodes (or nemas) or roundworms approach the bacteria and the blue green algae in ubiquity. They are found in the soil and in the water, and in the air as dust. In both marine and fresh waters and from the Arctic to the tropics.

2 Although the majority are free living, some occur as parasites of plants, animals, and man, and some of these parasites are among our most serious.

3 With this distribution, it is obvious that they will occasionally be encountered as plankton. A more complete discussion of nematodes and their public health implications in water supplies will be found elsewhere (Chang, S. L.).

E Additional crustacean groups sporadically met with in the plankton include the following:

1 Order Anostraca or fairy shrimps (formerly included with the two following orders in the Euphyllopoda) primarily planktonic in nature.

a Extremely local and sporadic, but when present, may be dominating.

b Artemia, the brine shrimp, can tolerate very high salinities.

c Very widely distributed, poorly understood.

2 Order Notostraca, the tadpole shrimps. Essentially southern and western in distribution.

3 Order Conchostraca, the clam shrimps. Widely distributed, sporadic in occurrence. Many local species.

4 Subclass Ostracoda, the seed shrimps. Up to 3 in. in length. Essentially benthic but certain species of *Cypris*, and *Notodromas* may occur in considerable numbers as plankton at certain times of the year.

5 Certain members of the large subclass Malacostraca are planktonic to some extent.

a The scuds, (order Amphipoda) are essentially benthic but are sometimes collected in plankton samples around weed beds or near shore.

b The mysid, or opossum shrimps are represented among the plankton by *Mysis relicta*, which occurs in the deeper waters, large lakes as far north as the Arctic Ocean.

F The Class Archnoidea, Order Hydracarina (or Acari) the mites. Not unusual in plankton tows near shore although Unionicola crassipes has been reported to be virtually planktonic.

G The phylum Mollusca is but scantily represented in the freshwater plankton, in contrast to the marine situation. Glochidia (ciliated) larval are occasionally collected, and snails now and then glide out on a quiet surface film and are taken in a plankton net,

H Eggs and other reproductive structures of many forms including fish, insects, and rotifers may be found in plankton samples. Special reproductive structures such as the statoblasts of bryozoa and sponges, and the ephippia of cladocerans may also be included.

I Adventitious and Accidental Plankters

Many shallow water benthic organisms may become accidentally and temporarily incorporated into the plankton. Many of those in the preceding section might be listed here, in addition to such forms as certain free living nematodes, small oligochaetes, and tardigrades. Collembola and other surface film livers are also taken at times but should not be mistaken for plankton.

Pollen from terrestrial or aquatic plants is often unrecognized, or confused with one of the above.

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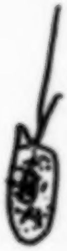
This outline was prepared by H. W. Jackson, Chief Biologist, FWPCA Training Activities, SEC.

Phylum PROTOZOA

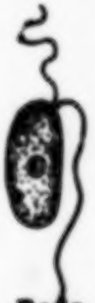
3/4

Free Living Representatives

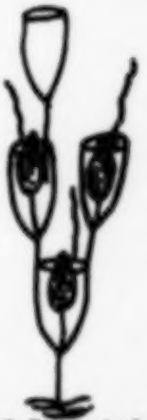
I. Flagellated Protozoa, Class Mastigophora



Anthophysis
Pollution tollerant
6 μ

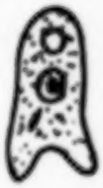


Bodo
Pollution tollerant
19 μ

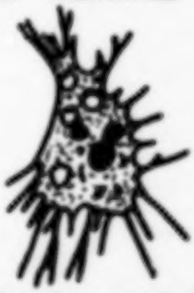


Colony of **Poteriodendron**
Pollution tollerant, 35 μ

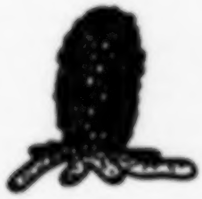
II. Ameboid Protozoa, Class Sarcodina



Dimastigamoeba
Pollution tollerant
10-50 μ

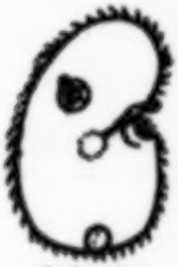


Nuclearia, reported
to be intollerant of
pollution, 45 μ.

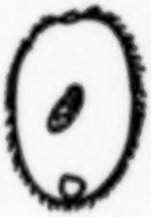


Difflugia
Pollution tollerant
50-500 μ

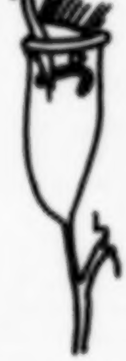
III. Ciliated Protozoa, Class Ciliophora



Colpoda
Pollution tollerant
20-120 μ



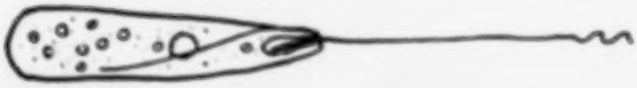
Holophrya, reported
to be intollerant of
pollution, 35 μ



Epistylis, pollution
tollerant. Colonies often
macroscopic.

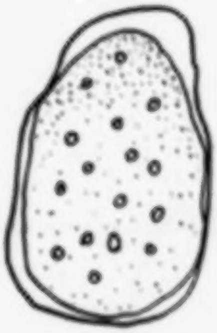
H.W.Jackson

PLANKTONIC PROTOZOA

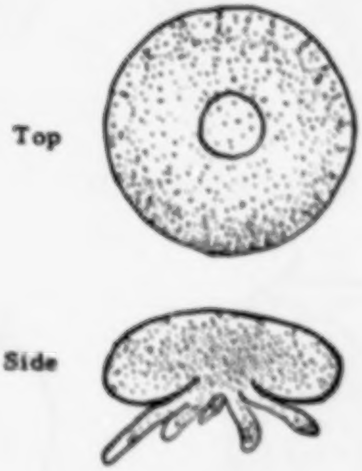


Peranema trichophorum

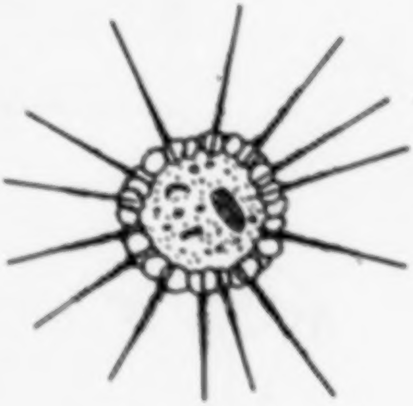
Reproduced from best available copy.



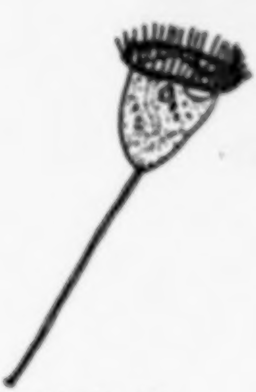
Chaos



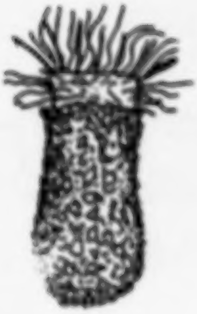
Arcella vulgaris



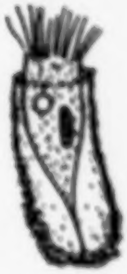
Actinosphaerium



Vorticella

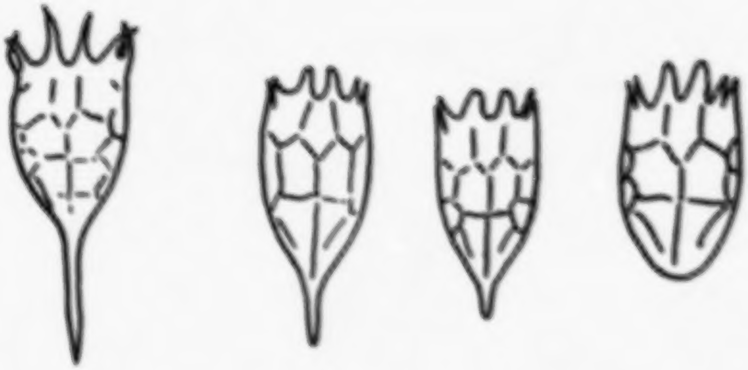


Codonella cratera

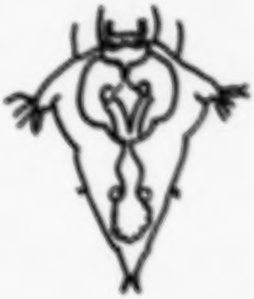


Tintinnidium fluviatle

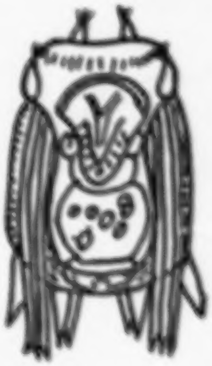
PLANKTONIC ROTIFERS



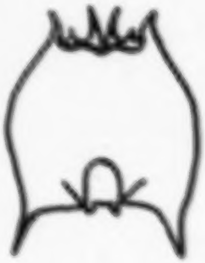
Various Forms of Keratella cochlearis



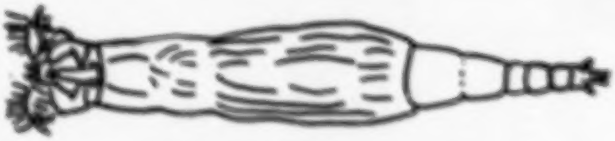
Synchaeta pectinata



Polygarthra vulgaris



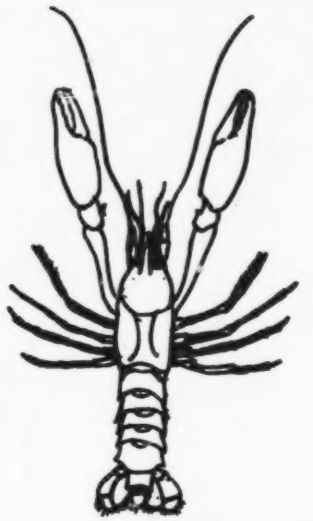
Brachionus quadridentata



Rotaria sp

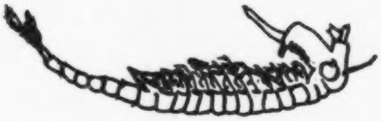
Class CRUSTACEA

3/4



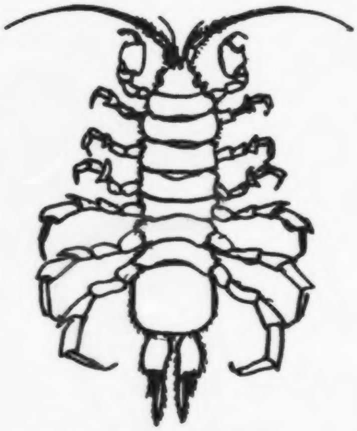
Crayfish, or crawdad; Cambarus, Order Decapoda

10-20 cm



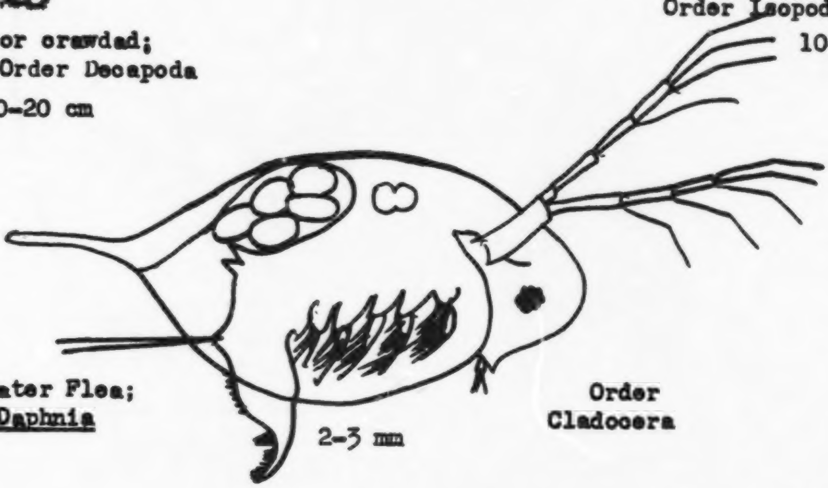
Fairy Shrimp; Eubranchipus, Order Phyllopoda

20-25 mm



Sow Bug; Asellus, Order Isopoda

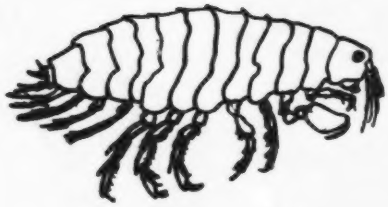
10-20 mm



Water Flea; Daphnia

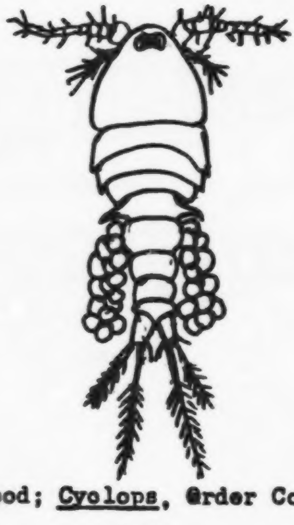
2-3 mm

Order Cladocera



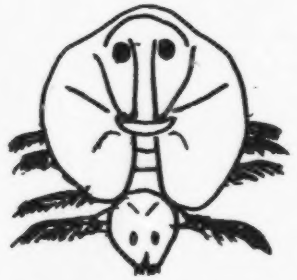
Scud; Hyalella Order Amphipoda

10-15 mm



Copepod; Cyclops, Order Copepoda

2-3 mm



Fish Louse, Argulus; a parasitic Copepod

5-6 mm

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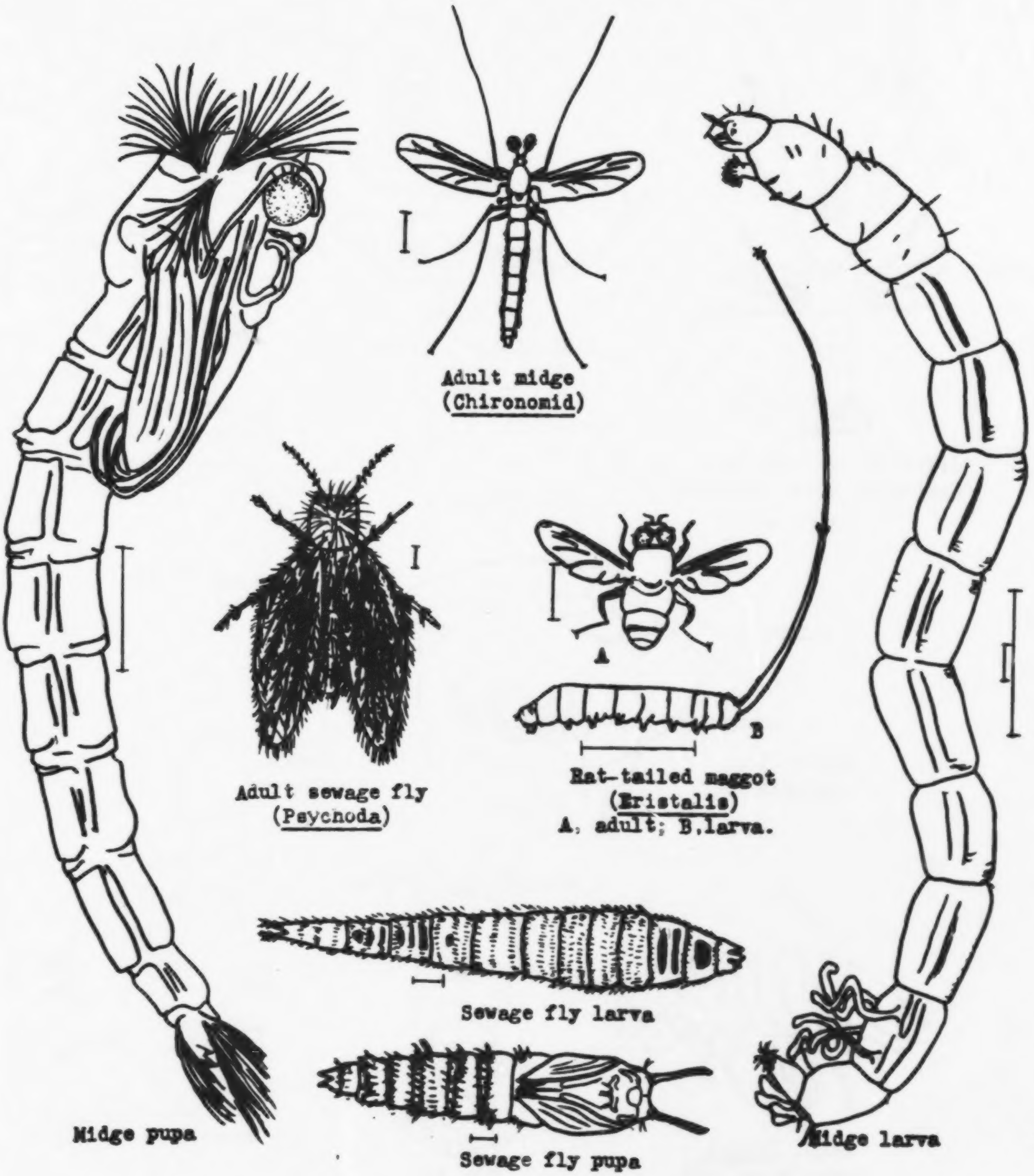
Two Winged Flies
Order DIPTERA

2/4



Adult midge
(Chironomid)

Adult sewage fly
(Psychoda)

Rat-tailed maggot
(Eristalis)
A, adult; B, larva.

Sewage fly larva

Sewage fly pupa

Midge pupa

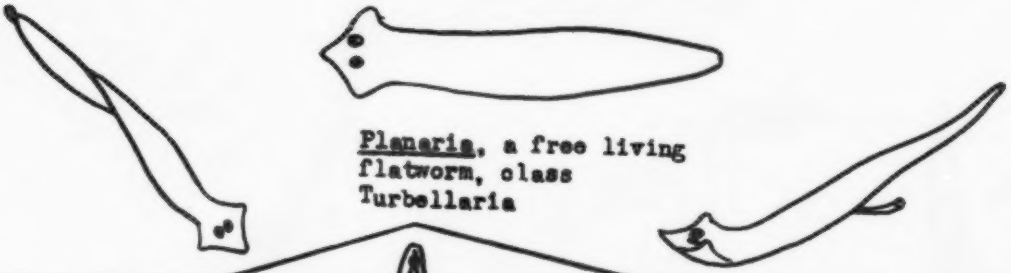
Midge larva

H.W.Jackson
After various authors

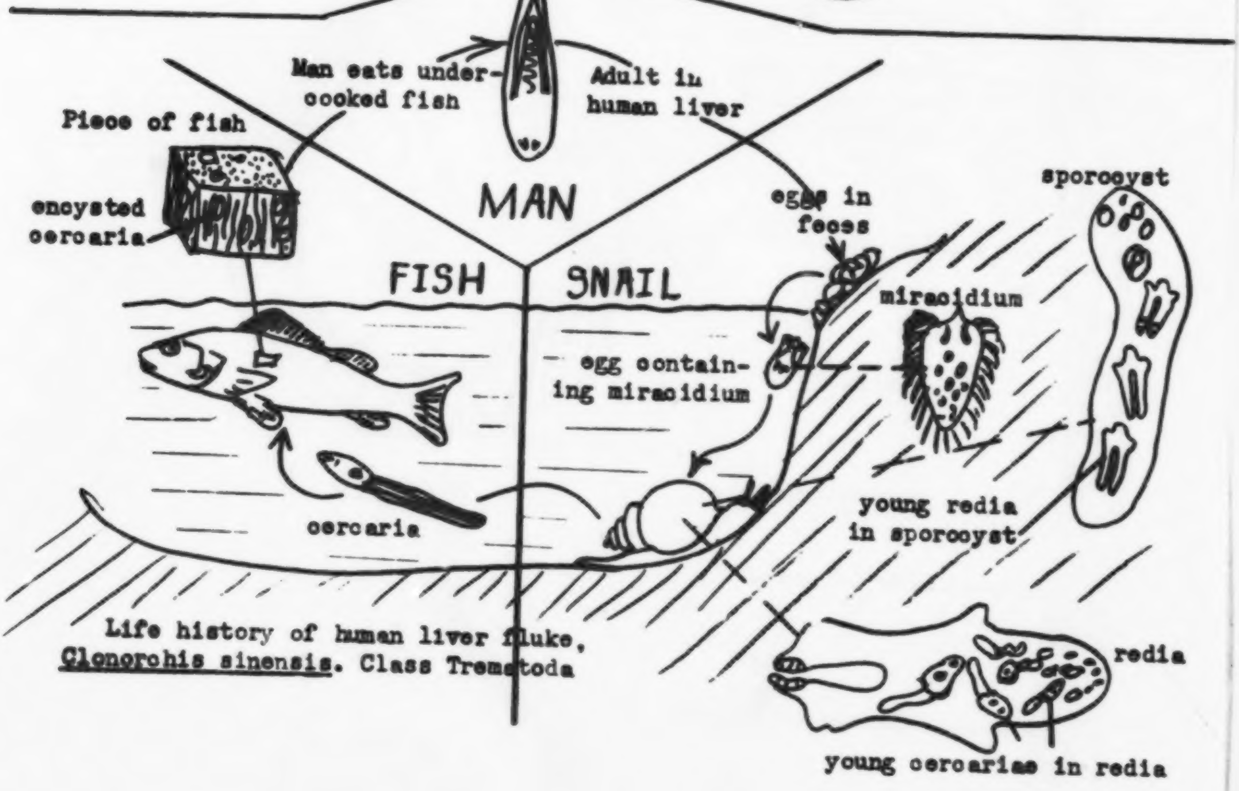
Flatworms

Phylum PLATYHELMINTHES

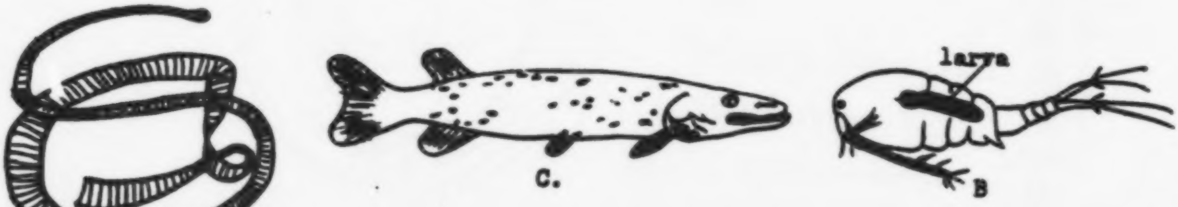
3/4



Planaria, a free living flatworm, class Turbellaria


Man eats under-cooked fish
Adult in human liver
Piece of fish
encysted cercaria
MAN
FISH
SNAIL
eggs in feces
sporocyst
miracidium
egg containing miracidium
young redia in sporocyst
cercaria
redia
young cercariae in redia

Life history of human liver fluke, *Clonorchis sinensis*. Class Trematoda



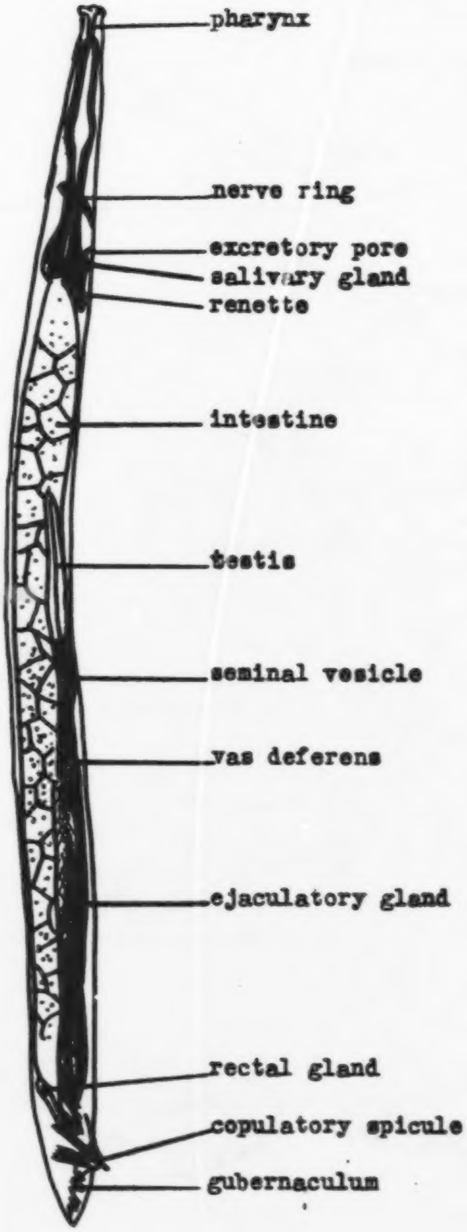
Aspects in the life cycle of the human tapeworm *Diphyllobothrium latum*, class Cestoda. A, adult as in human intestine; B, procercoid larva in copepod; C, plerocercoid larva in flesh of pickerel (X-ray view).

H.W.Jackson

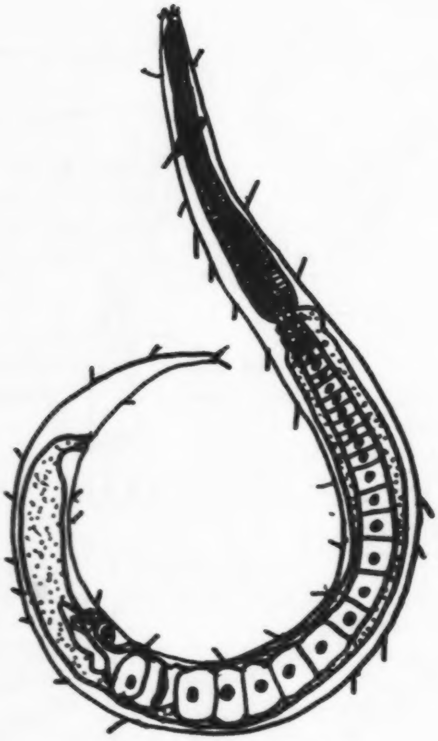
11/3

FREE LIVING NEMATHELMINTHES, OR ROUND WORMS

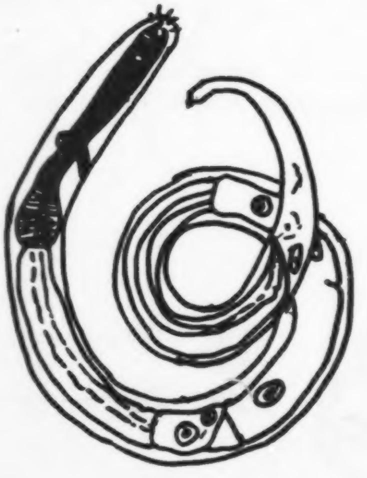
(For Administrative Use)


pharynx
nerve ring
excretory pore
salivary gland
renette
intestine
testis
seminal vesicle
vas deferens
ejaculatory gland
rectal gland
copulatory spicule
gubernaculum

Rhabditis ♂



Monhystera ♀

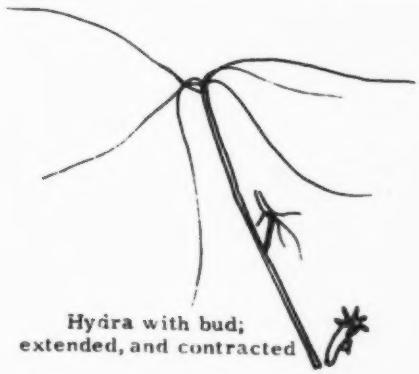


Achromadora ♀

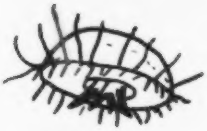
H.W.Jackson
(After Pennak)

MINOR PHYLA

Phy [illegible]

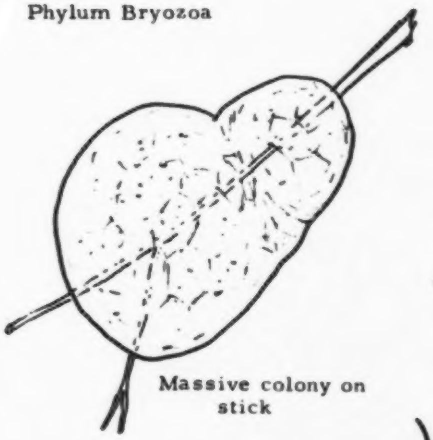


Hydra with bud;
extended, and contracted

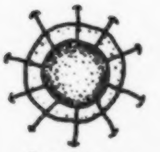


Medusa of
Craspedacusta

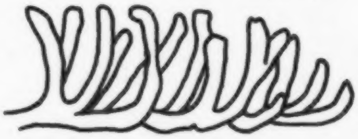
Phylum Bryozoa



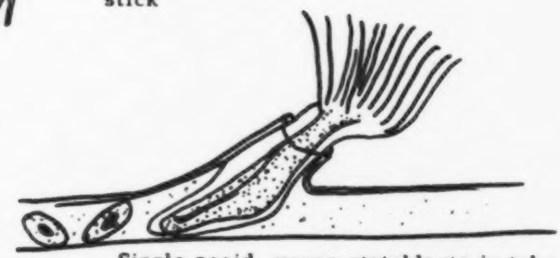
Massive colony on
stick



Statoblast



Creeping colony
on rock



Single zooid, young statoblasts in tube

INDICATOR-PATHOGEN RELATIONSHIPS IN TRIBUTARY STREAMS

R. M. Twedt*

I INTRODUCTION

A The establishment of water quality standards are a direct means of protecting public health and welfare. (1)

1 The "coliform group," defined by biochemical and physical procedures, has been used as an indicator of pollution since described by Escherich in 1885. (2)

2 Efforts have been directed toward increasing the significance of the coliform association with human waste.

a Parr devised IMViC Series (3)

b Elevated temperature test of Eijkman(4)

3 Geldreich et al established the significant correlation of elevated temperature test and Parr IMViC reactions for fecal coliforms. (5)

B Fecal streptococci have been used to measure animal pollution more accurately.(6)

1 Associations between fecal source and biochemical reactions have been noted. (7)

2 An analysis of FC/FS in polluted water will illuminate source as well as significance of pollution. (8)

C No quantitative relationship has been established between indicator organisms and pathogenic Salmonella in tributary streams.

1 Pollution indicator concentration always open to question.

2 Only limited semi-quantitative data is available. (9, 10)

II

The objectives of the Michigan study are to characterize the quantitative relationships existing between densities of fecal pollution indicators and pathogenic Salmonella in all environments found in tributary streams.

III THE PROFILE STUDY:

A determination of naturally occurring relationships of bacterial and invertebrate indicators and Salmonella in stream samples.

A Methods

1 Membrane filter procedures for TC, FC, FS

2 MPN multiple tube dilution method using dulcitol-selenite enrichment of Raj(10) to MacConkey's and XLD Agar Taylor secondary plates. (11)

3 Chemistry included pH, Temp. DO, BOD.

4 Analysis of distribution of aerobic and anaerobic ancylid mollusks on stream vegetation.

B Results and Discussion

1 The Rouge River: Evans Branch

2 The Saline: a tributary of the Raisin

3 The Huron: a tributary of Detroit River.

4 Salmonella isolation under conditions of:

a FC/FS 1.0

b FC 1000/100 ml

c Absence of ancylid mollusks

IV PROJECTED STUDIES

A The influence of toxic chemical elements on the indicator-pathogen relationship.

B The indicator-pathogen relationship during survival in water.

1 Natural microbial relationships

2 Seeded samples

3 Samples suspended in tributary streams in dialysis bags.

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CHLORINATION AND OTHER DISINFECTION PRACTICES

Joseph M. Dennis*

I INTRODUCTION

A Disinfection of drinking water with chlorine gas was first attempted at Louisville, Kentucky by George W. Fuller in 1896.(1)

B By 1914, 800 million gallons of water per day was being disinfected with chlorinated lime or liquid chlorine for public use. By 1948 an estimated 10,000 mgd of water was being chlorinated in more than 7000 American communities. It has been recently estimated that 76,000,000 people or 57% of the American population is being served by systems which are certified by the Surgeon General as meeting the Public Health Service Drinking Water Standards.(2) Most of these supplies use chlorination for disinfection.

C Drinking water, if properly treated, contains minimum numbers of bacteria, but as an extra measure of safety we recommend water, used by interstate carriers, be disinfected by any proven effective means or method. Chlorine is the most practiced disinfectant used today for drinking water supplies, mainly because of its excellent bacteriocidal effects and its residual can be easily measured in the water.

D Other disinfectants have been used such as chlorine dioxide, ozone, ultra violet light, chloramines, bromine and silver compounds. These methods and modifications are used mainly to alleviate taste and odor situations caused by industrial wastes and flora or fauna in the raw water. Since none of these disinfectants are in general use, we will limit this lecture to chlorination only:

II CHLORINATION:

Chlorination of public water supplies is the most important process used today in the production of water of a safe, sanitary quality.

Disinfection refers to the reduction of bacterial populations to a safe level, as contrasted to sterilization which refers to the total distruction of the bacterial population.

Chlorine for disinfection can be used in the dry, liquid or gaseous form. Compounds such as chloride of lime, sodium hypochlorite, etc., all have chlorine as the active disinfectant.

Under conditions of normal pressure and temperature chlorine exists as a gas, but it can be easily liquified for shipment in 100 lb. to 30 ton steel containers.

Chlorine is deadly poison and should be handled accordingly. Gas masks must be provided outside the chlorine storage room as a safety precaution. Wet chlorine is quite corrosive to iron and steel, therefore one should guard against the possibilities of leaks in the feeding apparatus.

The elements of the chlorine feeding apparatus are shown in the accompanying diagram.(3) They consist of a chlorine pressure reducing and regulating valve, rate of flow controller and indicator, and an injection device.

A Factors which influence the effectivenesss of disinfection of natural waters with chlorine will depend upon:

1 The suspended solids that may shield bacteria from the destructive action of chlorine.

2 The ammonia, either naturally present or added during the treatment processes, which reacts with the free available chlorine to form less effective disinfectants.

3 The organic matter in the water that may react with the chlorine and form compounds that have no disinfecting capabilities.

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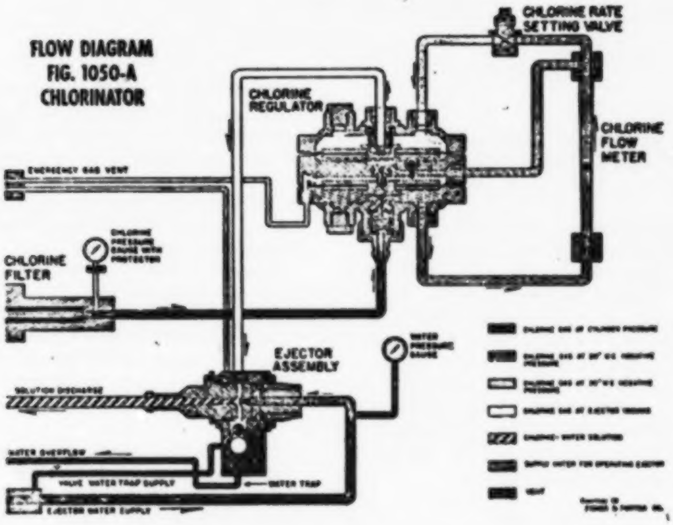
FLOW DIAGRAM
FIG. 1050-A
CHLORINATOR
CHLORINE RATE SETTING VALVE
CHLORINE REGULATOR
CHLORINE FLOW METER
EMERGENCY GAS VENT
CHLORINE FILTER
EJECTOR ASSEMBLY
WATER PRESSURE GAUGE
SOLUTION DISCHARGE
WATER OVERFLOW
VALVE WATER TRAP SUPPLY
WATER TRAP
EJECTOR WATER SUPPLY

FIG. 22 CHLORINATOR FLOW DIAGRAM (F&P)

4 The time of contact, pH, temperature and concentration of the residual chlorine in the water to be treated.

B Chlorine Disassociation

When chlorine is dissolved in water it disassociates as shown below. These are reversible reactions. The percentage of each ion or compound in the water is dependent upon the pH.

$$\underset{}{Cl_2 + H_2O} \rightleftharpoons \underset{\text{Hypochlorous Acid}}{HOCl} + HCl$$

$$HOCl \rightleftharpoons H^+ + \underset{\text{Hypochlorite ion}}{OCl^-}$$

At pH 6 to 7 the percentage of Hypochlorous Acid is between 80 and 95%. At pH 7 to 8 the percentage of HOCl is between 30 and 80%, while above pH 9 the HOCl is reduced to less than 5%. HOCl is a much stronger disinfectant than the OCl^- ion.

Free available residual chlorine is defined as the residual chlorine existing in water as hypochlorous acid and the hypochlorite ion. Combined available residual chlorine is that chlorine existing in water in chemical combination with ammonia or organic nitrogen compounds.

If reliance is to be placed on combined available chlorine, which is a weak disinfectant, a greater concentration, acting over a longer time must be provided. Experience has shown that a free available chlorine residual of 0.05 ppm with a reaction time of 10 minutes at pH 7.0 will result in the same bacterial kill as a combined available chlorine residual of 0.6 ppm with a reaction period of 60 minutes.

At pH of 6.5 and a temperature of 70°F., 0.3 ppm of combined residual causes a 100% bacterial kill in 60 minutes. With the same temperature at pH 7.0 it takes a concentration 0.6 ppm to effect the same kill and at 8.5 the concentration must be increased to 1.2 ppm.

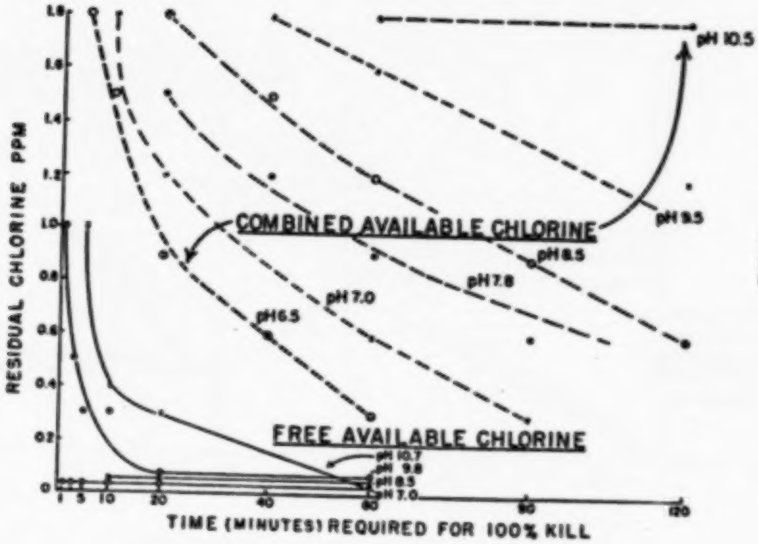

FIG. 26 - EFFECT OF KIND OF RESIDUAL AND TIME

Disinfection follows somewhat the laws of "chemical reaction." Therefore one expects the efficiency of disinfection would increase with the temperature.

It is thought that chlorine destroys bacteria by reacting with the proteins and ammino groups of the cell wall structure. This action alters the chemical characteristics of the cell contents and destroys life, or may cause complete disintegration of the shell structure.

C Advantages of Free Residual Chlorination

At times it becomes necessary to use other than free chlorine as a disinfecting agent. Many of the reasons given by the proponent are either invalid or a method of "one pulling himself up by his own boot straps." In summary some of the advantages of free chlorine residuals are:

1. A free residual will effect the same kill in 10 minutes that would take 60 minutes were the chlorine combined, say with ammonia.
2. No change is required in the free chlorine dosage from pH 6.0 to 8.0 to effect the same percentage kill, while with a combined chlorine, the dosage must be increased as the pH increases.
3. Little increase is required in the free chlorine dosage with a decrease in temperature while combined chlorine dosages must be increased markedly.
4. With some waters there is a distinct improvement insofar as taste and odors are concerned if one can maintain a free chlorine residual.
5. Many viruses that are not detectable by the ordinary laboratory procedures are resistant to combined chlorine disinfectants. (4)

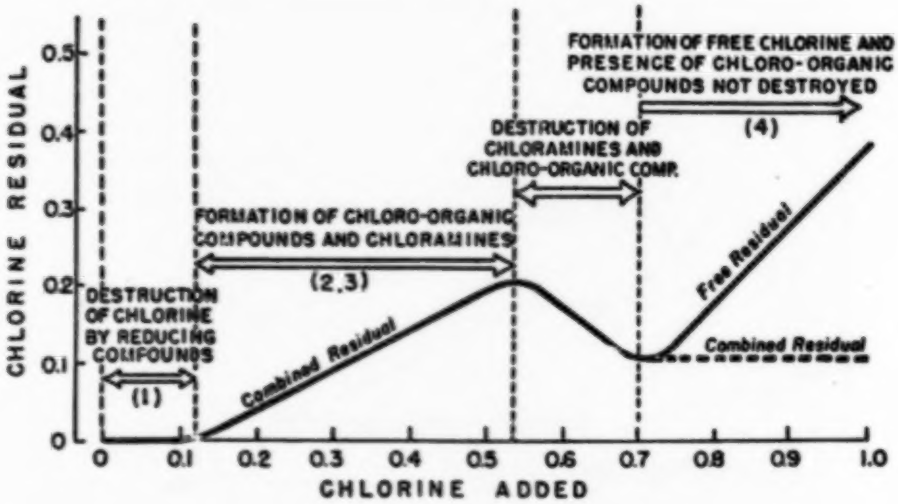

FIG. 25 - REACTIONS OF CHLORINE IN WATER

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Exercise I

MPN PRESUMPTIVE AND CONFIRMED TEST

A EQUIPMENT AND MATERIALS

Lauryl Tryptose Broth
Brillant Green Bile Broth
Buffered Dilution Blanks
EC Broth
Nutrient Agar Slants
EMB Agar-Levine
Innoculating Loop
Azide Dextrose Broth
Ethyl Violet Azide Broth
Gram Stains
Microscope

B PROCEDURE

The instructions listed in "1" below shall be done simultaneously in both Lauryl Tryptose Broth for coliform bacteria and Azide Dextrose Broth for fecal strep.

1 Presumptive Test

a Fill out laboratory work sheet with appropriate information.

b Label tubes according to sample number and dilution.

c Shake sample bottle vigorously 25 times.

d Pipet 11 ml of sample into a 99 ml dilution blank. This constitutes a 1/10 dilution of the sample.

e Pipet 10 ml of sample into each of the 5 double strength presumptive broth tubes in row "1."

f Pipet 1 ml of sample into each of the 5 single strength presumptive broth tubes in row "2."

g Shake the bottle containing a 1/10 dilution of the sample vigorously 25 times and pipet 11 ml into a 99 ml dilution blank. This constitutes a 1/100 dilution of the sample.

h Pipet 1 ml of the 1/10 dilution into each of the five single strength broth tubes in row "3."

i Shake the bottle containing the 1/100 dilution vigorously 25 times and pipet 1 ml into each of 5 single strength presumptive broth tubes labeled row "4."

j Incubate all tubes at 35°C for 48 hours.

2 Confirmed Tests

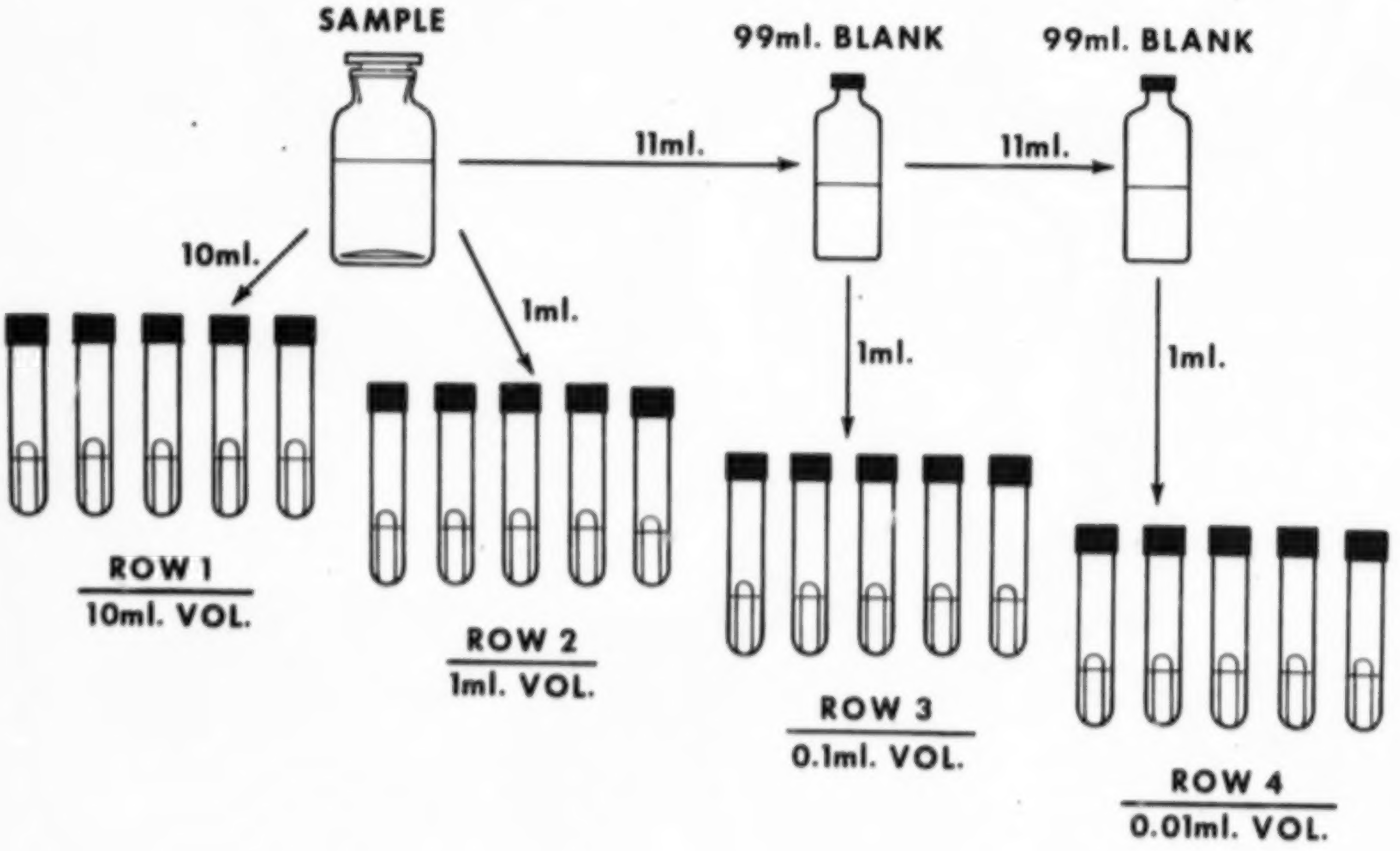
a Coliform Group

Using a sterile metal loop 3 mm in diameter, transfer one loopful of medium from all 24 and 48 hour gas positive tubes to fermentation tubes containing brilliant green lactose bile. The presence of gas in BGLB at any time within 48 (+ 3) hours constitutes a positive confirmed test. MPN results are computed from the gas positive tubes.

b Fecal Strep

Transfer 3 loopfuls of culture medium from all tubes of azide dextroxe broth showing turbidity to tubes containing 10 ml of sterile ethyl violet azide broth. The minimum diameter of the loop should be 3 mm. The presence of a purple button at the bottom of the EVA broth or dense turbidity at the end of a 48 hour incubation period at 35°C is a positive confirmed test for fecal strep. MPN results are computed from the number of confirmed tubes.

PREPARATION OF DILUTIONS FOR MPN PROCEDURE


SAMPLE
99ml. BLANK
99ml. BLANK
11ml.
11ml.
10ml.
1ml.
1ml.
1ml.
ROW 1
10ml. VOL.
ROW 2
1ml. VOL.
ROW 3
0.1ml. VOL.
ROW 4
0.01ml. VOL.

Sample No. ______________________ Name ______________________

Group ______________________

Date ______________________

DATA SHEET
Fermentation Tube Tests

ml sample	Coliform Test							Gram stain	Fecal coli-form	Fecal Streptococcus				Remarks
	LTB		BGLB		EMB	LSTB				A - D		EVA		
	24	48	24	48	24	24	48		24	24	48	24	48	

Coliform MPN/100 ml

Confirmed:

Completed:

Fecal Coliform MPN:

Fecal Streptococcus MPN/100 ml

A - D - EVA:

20-4 ·1

Exercise II

STANDARD PLATE COUNT

A EQUIPMENT AND MATERIALS

Plate Count Agar
11 ml. and 1 ml. Pipets
Samples #1 and #2
Dilution bottles
Glass or Plastic Culture Plates

B PROCEDURES

1 Set up two rows of three plates each, number and label with appropriate dilution: 1 ml, 0.1 ml, and 0.01 ml.

2 Shake sample number 1 vigorously 25 times.

3 Make a 1/10 dilution by adding 11 ml. sample to a 99 ml. dilution blank. Pipet 1 ml. of sample into the appropriate plate.

4 Shake a 1/10 dilution vigorously 25 times. Prepare 1/100 dilution by adding 11 ml. of 1/10 dilution to 99 ml. of buffer.

5 Plate 1 ml. of 1/10 dilution into appropriate plate.

6 Shake 1/100 dilution vigorously 25 times and pipet 1 ml. into appropriate plate.

7 Repeat the procedure with sample number 2.

8 Pour plates, mix gently and allow media to solidify. Pour 1 plate each for agar, buffer and air controls.

9 Incubate plates for 24 (±) 2 hours at 35°C or 48 (±) 3 hours at 20°C.

10 Count colonies with a counting aid such as a Quebec Colony Counter and record results on appropriate form.

Exercise III

COMPLETED AND FECAL COLIFORM TESTS

A EQUIPMENT AND MATERIALS

Lauryl Tryptose Broth
Nutrient Agar Slant
EMB Plate
EC Broth
Innoculating Loop
Gram Stains

B PROCEDURE

1 Monday

Each trainee shall pick one characteristic sheen colony from each of 2 membrane filters and streak on EMB plates. Innoculum from each of two gas positive BGLB tubes shall also be streaked on EMB plates. Incubate all four EMB plates at 35°C for 24 hours.

2 Tuesday

A single typical fish-eye type coliform colony from each of the four EMB plates will be innoculated onto a nutrient agar slant and into a lauryl tryptose broth fermentation tube.

3 Wednesday

Transfer 1 loopful of medium from each gas positive lauryl tryptose tube to a tube of EC broth and incubate in a 44.5°C water bath. Record positive or negative 24 hour results. The presence of gas in the fermentation tube constitutes a positive test for fecal coliforms.

4 Thursday

Prepare slides from each agar slant which correponds to a gas positive LTB tube and stain with the Gram Stain. Observe stained material under microscope and record as positive or negative for gram negative nonsporeforming rod forms. Read 48 hour lauryl tryptose tubes and record as positive or negative. The presence of gram (-), nonsporeforming, rod forms which produce gas in LTB or lactose broth in 48 hours constitutes a completed test for bacteria of the coliform group

Exercise IV

MEMBRANE FILTER LABORATORY PROCEDURES

A Equipment and Materials

m-Endo Agar (dehydrated powder preweighed for 50 ml in vial)
m-Enterococcus Agar (dehydrated powder preweighed for 50 ml in vial)
MFC Agar (dehydrated powder preweighed for 50 ml in vial)
Petri Dish, MF Sterile
Membrane, MF Sterile 0.45μ pore size
Incubator 35 Deg. C. + 0.5
Incubator 44.5 Deg. C. ± 0.5
Bag, plastic
Alcohol (EtOH)
Rosolic Acid (dissolved in 0.2 N NaOH)
Filtration Apparatus for MF
Forceps and MeOH (Methanol) sterlizing solution in bottle
*Blanks, Dilution (99ml)
*Water, Sterile Buffered, for Rinse of MF apparatus
Pipets Sterile
Burner, Bunsen
Microscope, stereoscopic
Counter, colony
Sheet, Data
Pencil, Marking

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*Items are interchangeable

B Procedure

1 Tuesday

a Preparation of Media

Each of the three mediums mentioned above are to be prepared and plates poured. Allow to harden before application of membranes which have been used for the various sample volumes as given in the laboratory instructions. It is imperative to prepare media without undue exposure to heat or direct flame. The addition of Ethanol to m-Endo Agar and Rosolic acid to MFC Agar is necessary for a complete medium.

b Sample Filtrations

Filtrations of the given sample volumes are to be made for each of the two samples provided in the wide mouth sample bottles. Sample 25 is a sewage treatment plant influent sample and sample 26 is a river water sample. Data sheets are to be initiated with pertinent data before any filtrations are made.

c Incubation

Incubation of the total coliform plates (m-Endo) and the fecal streptococci plates (m-Enterococcus) are to be made at 35 Deg. C. for 22 + 2 hours for the former and 48 + 3 hours for the latter. The MFC plates (fecal coliform) will be placed in plastic bags and immersed and kept under water for 24 hours in the 44.5 Deg C. water bath.

2 Wednesday

a Colony Counts

Colony counts are to be made for the total coliform (m-Endo) and the MFC (fecal coliform) plates. Inspect each of the plates starting from the highest dilution and record the numbers of characteristic colonies for each of the media Numbers which are well over a "countable" plate range are recorded as TNTC (too numerous to count) and those which have any plating errors or anomolies will be so marked.

b Recording of Data

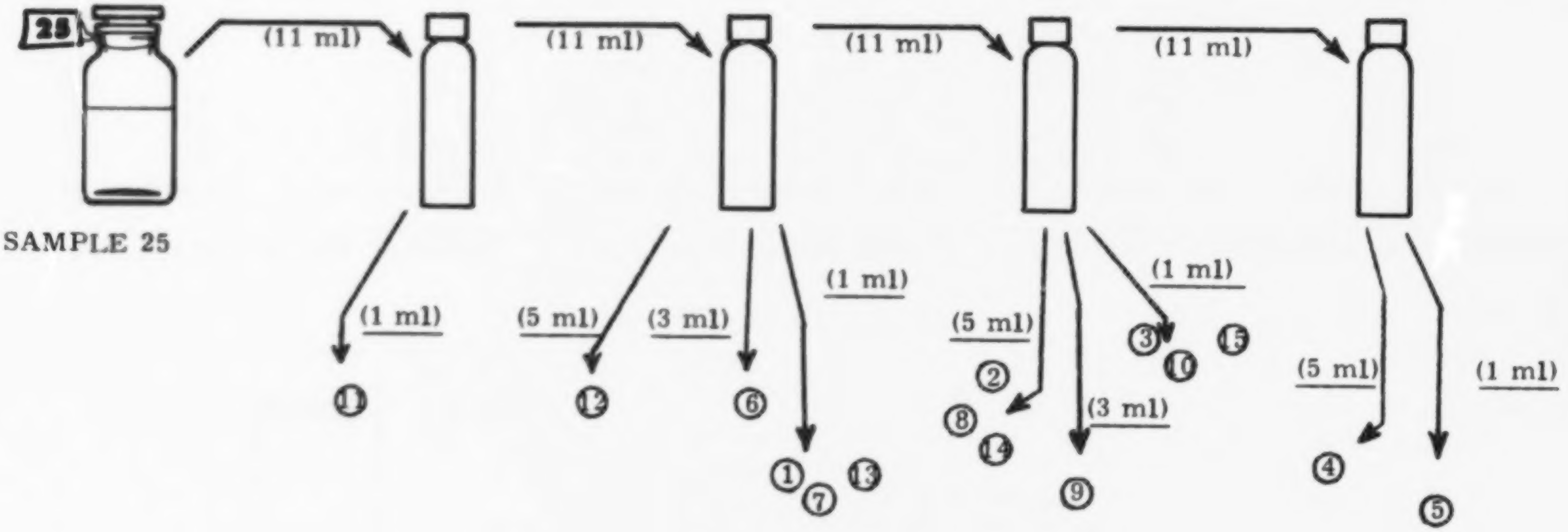
Plates within the countable plate ranges will be used to ascertain the counts/100 ml for the total coliform and fecal coliform organisms.

3 Thursday

Procedures will be followed as above to ascertain the count /100 ml for the fecal streptococci (m-Enterococcus Agar).

SAMPLE 25

Schematic for sample dilution
and sample volumes


SAMPLE 25
25
(11 ml)
(11 ml)
(11 ml)
(11 ml)
(1 ml)
(5 ml)
(3 ml)
(1 ml)
(5 ml)
(3 ml)
(1 ml)
(5 ml)
(1 ml)
⑪
⑫
⑥
①
⑦
⑬
②
⑧
⑭
⑨
③
⑩
⑮
④
⑤

TOTAL COLIFORM (m-Endo)		FECAL COLIFORM (MFC)		FECAL STREPTOCOCCI (m-Enterococcus)	
Plate No.	Sample Volume	Plate No.	Sample Volume	Plate No.	Sample Volume
1	0.01 ml	6	0.03 mi	11	0.1 ml
2	0.005	7	0.01	12	0.05
3	0.001	8	0.005	13	0.01
4	0.0005	9	0.003	14	0.005
5	0.0001	10	0.001	15	0.001

SAMPLE 26

Schematic for sample volumes

All sample volumes directly from sample bottle

- Use sterile graduate for volumes ≥ than 25.0 ml and pipet for others.

TOTAL COLIFORM (m-Endo)		FECAL COLIFORM (MFC)		FECAL STREPTOCOCCI (m-Enterococcus)	
Plate No.	Sample Volume	Plate No.	Sample Volume	Plate No.	Sample Volume
16	15.0 ml	21	20.0 ml	26	30.0 ml
17	10.0	22	15.0	27	25.0
18	5.0	23	10.0	28	15.0
19	3.0	24	5.0	29	8.0
20	1.0	25	2.0	30	5.0